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Host-specific parasites (*Dactylogyrus*, Monogenea) as indicator of evolution and historical dispersion of their cyprinid fish hosts in the Mediterranean region

Ph.D. Thesis

MICHAL BENOVICS

Supervisor: prof. RNDr. Andrea Vetešníková Šimková, Ph.D.
Department of Botany and Zoology

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Abstract

One of the fundamentals of evolutionary biology is the study of host-parasite coevolutionary relationships. Due to intertwined evolutionary history of two interacting species and on-going coadaptation processes of hosts and parasites we can expect that studying parasites will shed more light into evolutionary processes of their hosts. Monogeneans (ectoparasitic Platyhelminthes) and their fish hosts represent one of the best model for studying host-parasite evolutionary relationships using cophylogenetic approach. These parasites developed remarkably high host specificity, where each host species often serves as potential host for its own host-specific monogenean species.

This thesis is focussed on the host-parasite system of *Dactylogyrus* (gill monogeneans) and their freshwater fish hosts in the peri-Mediterranean region. *Dactylogyrus* are almost exclusively parasites of cyprinoid fish and exhibit remarkable species diversity. This diversity is the most likely linked with the high diversity of their fish hosts, where Cyprinoidei represent highly diverse freshwater fish taxon. However, the historical dispersion of the cyprinoids in the peri-Mediterranean is not clearly resolved and is far more complex than could be explained by single dispersal model. Therefore, in this Ph.D thesis, the *Dactylogyrus* parasites were used as additional tool to investigate evolutionary history of their hosts.

Communities of endemic *Dactylogyrus* in the peri-Mediterranean appear to be species poorer in comparison to Central Europe. However, their diversity is still weakly explored, as thesis eight new species were described in the Euro-Mediterranean region in this. Moreover, numerous species complexes appear to be present among *Dactylogyrus* parasites in the peri-Mediterranean, suggesting even higher species diversity of these monogeneans. Cophylogenetic methods revealed strong coevolutionary structure between phylogenies of *Dactylogyrus* and their respective cyprinoid hosts in peri-Mediterranean area with host switch as common coevolutionary event. Many host-parasite links significantly contributed into the global coevolutionary structure suggesting the strong coevolutionary link between associated hosts and parasites.

The results presented in this thesis demonstrated usefulness of investigating the host-specific parasites phylogenetic relationships for uncovering evolutionary relationships between host lineages. Moreover, host-specific *Dactylogyrus* were shown as good marker to reveal historical and even more recent contacts (detecting introduction of non-native fish hosts into new regions) between cyprinoid host lineages.

Abstrakt

Jedním ze základních stavebních kamenů evoluční biologie je studium parazito-hostitelských koevolučních vztahů. Jelikož evoluční historie parazitů a jejich hostitelů je obvykle úzce spjatá, je možné předpokládat, že studium parazitů přispěje k poznání evolučních procesů jejich hostitelů. Monogenea (Platyhelminthes) a jejich rybí hostitelé představují jeden z nejzajímavějších systémů pro studium parazito-hostitelských evolučních vztahů. U zástupců této skupiny parazitů se vyvinula velice úzká hostitelská specifita, kdy je jeden druh parazita často schopen cizopasit pouze na jednom druhu hostitele.

Předložená dizertační práce je zaměřena na parazito-hostitelský systém *Dactylogyrus* (žaberní ektoparazité) a jejich rybí hostitele v Mediteránní oblasti. Studovaný rod cizopasníků se vyznačuje vysokou druhovou diverzitou a jednotliví zástupci jsou téměř výhradně paraziti kaprovitých ryb. Fascinující druhová diverzita parazitů rodu *Dactylogyrus* je s největší pravděpodobností důsledkem druhové bohatosti kaprovitých ryb, neboť tato skupina představuje nejvíce diverzifikovanou skupinu mezi sladkovodními rybami. Evoluční historie a historická disperze kaprovitých ryb v Mediteránu však není zcela objasněna a je pravděpodobně složitější, než se na první pohled může zdát. Právě proto se v této dizertační práci využívají paraziti jako nástroj pro objasnění evoluční historie rybích hostitelů.

Ve všeobecnosti se jeví, že společenstva zástupců rodu *Dactylogyrus* jsou ve srovnání se střední Evropou druhově chudší v oblasti Mediteránu. Avšak celková diverzita parazitů je evidentně i nadále málo prozkoumaná, jelikož v rámci řešení problematiky dizertační práce bylo popsáno osm nových druhů *Dactylogyrus*. Navíc, molekulární analýzy naznačují existenci mnoha komplexů kryptických druhů, které mohou být klasickým taxonomickým přístupem, založeném na morfologii, jenom těžko identifikovatelné. Kofylogenetické metody odhalily statisticky významnou koevoluční strukturu mezi evolučními historiemi parazitů rodu *Dactylogyrus* a jejich rybími hostiteli. Více než polovina paraziticko-hostitelských asociací významně přispívala k celkové kofylogenetické struktuře.

Výsledky předložené práce demonstrují užitečnost hostitelsko-specifických parazitů pro účely studia evoluční historie a biogeografie jejich hostitelů. Navíc, studiem parazitů rodu *Dactylogyrus* je možné detekovat historické kontakty mezi evolučně vzdálenými liniemi ryb, které recentně žijí v alopatrii.

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The last, but not least, sincere gratitude and all my respect goes to my colleagues who shared the office with me and provided all-necessary distraction during the course of study. And of course I have to thank my grandmother for all her support, despite that she still have no idea what I am doing with my life.

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Original publications linked to the thesis

The results of presented Ph.D thesis are composed of seven research papers. Papers are ordered chronologically by publication or submission date.

Paper I

Benovics M., Kičinjaová M. L. & Šimková A. (2017) The phylogenetic position of the enigmatic Balkan *Aulopyge huegelii* (Teleostei: Cyprinidae) from the perspective of host-specific *Dactylogyrus* parasites (Monogenea) with a description of *Dactylogyrus omenti* n. sp. *Parasites & Vectors* 10: 547. doi: 10.1186/s13071-017-2491-z [IF_{2017} = 3.031, Q1]

MB actively participated on the processing of fish and parasitological material, identified parasite species, performed all laboratory procedures and phylogenetic analyses, and wrote the manuscript. Overall contribution = ~70%.

Paper II

Šimková A., **Benovics M.**, Rahmouni I. & Vukić J. (2017) Host-specific *Dactylogyrus* parasites revealing new insights on the historical biogeography of Northwest African and Iberian cyprinid fish. *Parasites & Vectors* 10: 589. doi: 10.1186/s13071-017-2521-x [IF_{2017} = 3.031, Q1]

MB actively participated on the processing of fish and parasitological material, and identification of parasites species collected during field work. He participated on data analyses. Overall contribution = ~10%.

Paper III

Benovics M., Desdevises Y., Vukić J., Šanda R. & Šimková A. (2018) The phylogenetic relationships and species richness of host-specific *Dactylogyrus* parasites shaped by the biogeography of Balkan cyprinids. *Scientific Reports* 8: 13006.
doi: 10.1038/s41598-018-31382-w [IF_{2018} = 4.011, Q1]

MB actively participated on the processing of fish and parasitological material, identified parasite species, performed all laboratory procedures for parasites, conducted phylogenetic analyses, and wrote the manuscript. Overall contribution = ~70%.

Paper IV

Benovics M., Desdevises Y., Šanda R., Vukić J. & Šimková A. (2020) Cophylogenetic relationships between *Dactylogyrus* (Monogenea) ectoparasites and endemic cyprinoids of the north-eastern European peri-Mediterranean region. *Journal of Zoological Systematics and Evolutionary Research* 58: 1–21. doi: 10.1111/jzs.12341 [IF_{2018} = 2.268, Q1]

MB actively participated on the processing of fish and parasitological material, identified parasite species, performed all laboratory procedures for parasites, conducted phylogenetic and cophylogenetic analyses, and wrote the manuscript. Overall contribution = ~60%.

Paper V

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* (in press).

doi: 10.1017/S0031182020000050 [IF_{2018} = 2.456, Q2]

MB actively participated on the processing of fish and parasitological material, identified parasite species, performed all laboratory analyses for parasites, conducted phylogenetic analyses, and wrote the manuscript. Overall contribution = ~70%.

Paper VI

Řehulková E., **Benovics M.** & Šimková A. (submitted) Seven new species of *Dactylogyrus* Diesing, 1850 (Platyhelminthes: Monogenea) from the gills of endemic cypriniform fishes in the Balkan Peninsula: an integrated morphological and molecular approach to species delimitation. *Parasitology Research* (November 2019, under the second review). [IF_{2018} = 2.067, Q2]

MB actively participated on the processing of fish and parasitological material, identified parasites species, performed phylogenetic analyses, and contributed to manuscript writing. Overall contribution = ~20%.

Paper VII

Benovics M., Vukić J., Šanda R. & Šimková A. (submitted) Disentangling the evolutionary history of peri-Mediterranean cyprinids using host-specific *Dactylogyrus* ecto-parasites (Monogenea: Monopisthocotylea). *Evolution* (January 2020, under review). [IF_{2018} = 3.573, Q1]

MB processed fish, collected parasitological material, identified parasite species, performed all laboratory procedures, conducted phylogenetic and cophylogenetic analyses, and wrote the manuscript. Overall contribution = ~70%.

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1 Introduction

From the biological, evolutionary, and ecological points of view parasites and their hosts represent completely different types of organisms. However, due to their on-going reciprocal co-adaptations over long period of time, these two interacting partners have developed rather intimate relationships. The parasitism is undoubtedly one of the most frequent life strategy in living organisms. The constant pressure of newly developed defence mechanisms by host (e.g. behavioural, ecological or immunological) to get rid of the unwelcome inhabitant, drives parasite to develop resistance to host attack just to avoid extinction in the hostile environment. These reciprocal dynamic coevolutionary interactions are called “arms race”. Such arms race may last over long period of time and impact the relative rate of evolution in both interacting partners. Therefore, the Fahrenholz’s rule was postulated, claiming that evolutionary history of the parasite taxa and their respective host taxa may actually mirror each other. This is partially true in the very specific host-parasite systems; however, the real degree of congruency of host and parasite evolution is usually influenced by multitude of factors (e.g. life cycle of parasite, host specificity of parasite, or dispersal capacities of both parasites and their hosts).

Monogeneans (Platyhelminthes) and their vertebrate hosts represent interesting host-parasite systems. Despite that numerous ectotherm vertebrate taxa (and a single endotherm species) may serve as potential hosts for monogeneans, these parasites are in general highly host-specific (however, there are also some generalist species parasitizing a wide range of host species). Hence, monogeneans appear to be the optimal proxies to investigate cophylogenetic host-parasite relationships due to their incredibly high species diversity, direct life cycle, and frequently high degree of host specificity.

Several host-parasite systems including fish in the position of host, and monogeneans in the position of parasites were previously studied to infer host-parasite coevolutionary relationships. Nevertheless, the most speciose genus, *Dactylogyrus* Diesing, 1850 still offers much to uncover in the regards to their Cyprinoidei hosts (term Cypriniodei is used here following the most recent classification), as this host-parasite system was investigated only in the central Europe. The remarkable species diversity of *Dactylogyrus* is putatively connected to their relatively high degree of host specificity and high variety to adaptation to their highly diversified cyprinoid hosts. Considering that each cyprinoid species may serve as host for at least one *Dactylogyrus* species (but in the majority of already investigated cyprinoid hosts the infection by more than one *Dactylogyrus* species is documented) we can assume that *Dactylogyrus* diversity is still underexplored. Whereas cyprinoids are strictly freshwater fish and therefore, they are intolerant to saline environment, several hypotheses have been proposed to explain their current biogeographical distribution in the peri-Mediterranean. Each one is connected with the different historical dispersion route, e.g. the northern route via river captures through Europe, the southern route through North Africa, or via Mediterranean basin during Lago Mare phase of Messinian salinity crisis. However, fossil records in the combination with molecular data suggest that the differentiation of Mediterranean fish taxa is far more complex than may be explained using single dispersal model.

Thus, *Dactylogyrus* parasites appear to be a good candidate to shed more light into historical dispersion and phylogeny of their cyprinoid hosts in the Mediterranean. Moreover, these parasites may serve as additional tool to uncover historical or more recent contacts between evolutionary and geographically distant fish lineages.

2 Aims of the study

1. to investigate *Dactylogyrus* species diversity on the endemic cyprinoid fishes in the peri-Mediterranean region
2. to reconstruct the evolutionary history of endemic *Dactylogyrus* and their cyprinoid hosts in the selected areas of the Mediterranean
3. to perform cophylogenetic analyses to reveal the speciation patterns in *Dactylogyrus* and to assess degree of congruency between phylogenies of *Dactylogyrus* parasites and their endemic cyprinoid hosts
4. to use the *Dactylogyrus* parasites of different highly diversified cyprinoid genera to investigate processes linked to the evolution and biogeography of endemic cyprinoids

3 Literature overview

3.1 Host-parasite coevolution and host specificity of parasites

3.1.1 Host-parasite coevolution

Parasitic life strategy evolved in multiple, highly divergent groups of organisms and represents in nature the most prevalent interspecific interaction with a driving force in ecology and evolution (Windsor, 1998, Poulin & Morand, 2000, 2004, Dobson et al., 2008). Over the long evolutionary time parasites adapted to their hosts and therefore the biology of interacting host and parasite is intertwined. As a result of continuous reciprocal coadaptation (also referred as “arms race model”, Dawkins & Krebs (1979), Ridley & Jones (2004)), evolution of parasite taxa leads to the increase of host specificity, i.e. highly adapted parasite species infects a single host species (Poulin, 2007).

Therefore, if the host specificity of given parasite taxa is high, we can expect on-going host-parasite cospeciation, observable as phylogenies of the hosts and the parasites are mirroring each other (postulated as Fahrenholz rule, Fahrenholz, 1913, Stammer (1957), Dogiel (1964), Klassen (1992), Brooks & McLennan (1993)). However, such coevolution is in nature rather rare and may be observed only in very specialized host-parasite systems (e.g. Hafner & Nadler, 1988, Hafner et al., 1994, Clayton & Johnson, 2003, Hosokawa et al., 2006). Besides cospeciation, there are other coevolutionary events playing an important role in the evolutionary history of parasites (Page, 1994, Page & Charleston, 1998). Observed incongruencies between host and parasite phylogenies may be generated by a wide range of processes, from which host switching between either phylogenetically close host lineages, or phylogenetically unrelated hosts living in the sympatry is the most notable (Brooks & McLennan, 1991, Klassen, 1992, Page, 2003). Even if frequent host switching may promote incongruences in the phylogenies, a series of multiple host switches, followed by parasite speciation may in contrary generate the similar topologies of host and parasite trees (de Vienne et al., 2007). Next coevolutionary event resulting in incongruences of host and parasite phylogenies is the extinction of the parasite in a host lineage during the evolutionary time (i.e. parasite is absent on the recent host lineage), or so-called “missing the boat” (i.e. speciation of host was not followed by speciation of parasite). In such case, usually parasite is not present in one of two separating host populations – this separation represents the initial step of host speciation. These two coevolutionary events are also referred as “sorting events” (Page, 1994, 2003, Johnson et al., 2003). Parasite taxa may also fail to diverge during the speciation process of the host and therefore, the same parasite species might be present on two recent, phylogenetically close host lineages (Paterson & Banks, 2001, Johnson et al., 2003, Page, 2003). Finally, due to the usually shorter generation time and faster mutation rate of the parasites, common cophylogenetic event, potentially leading into incongruences between host and parasite phylogenies, is intra-host speciation of parasites i.e. parasite duplication (Ronquist, 1997, Johnson et al., 2003, Page, 2003, de Vienne et al., 2013). Scenarios of coevolutionary events in host-parasite systems are presented in Figure 1.

Nonetheless, the final degree of congruency between host and parasite phylogenies and strength of interaction between these two taxa is influenced by many factors which might promote individual coevolutionary processes (further reviewed in Niebering & Olivieri, 2007). Besides of host specificity, the complexity of life cycle is the most notable parasite trait affecting host-parasite coevolution. More specifically, the intermediate hosts may serve as additional vector providing dispersion opportunity for parasite (Page, 2003, Wickström et al., 2003, Criscione et al., 2006). The presence of free-living stage in the parasite cycle may further promote host switching if the most suitable, i.e. typically associated, host is not present, which may generate additional incongruences in the host and parasite phylogenies (Blouin et al., 1995, Huyse & Volckaert, 2005, Bakke et al., 2007, Kritsky et al. 2011).

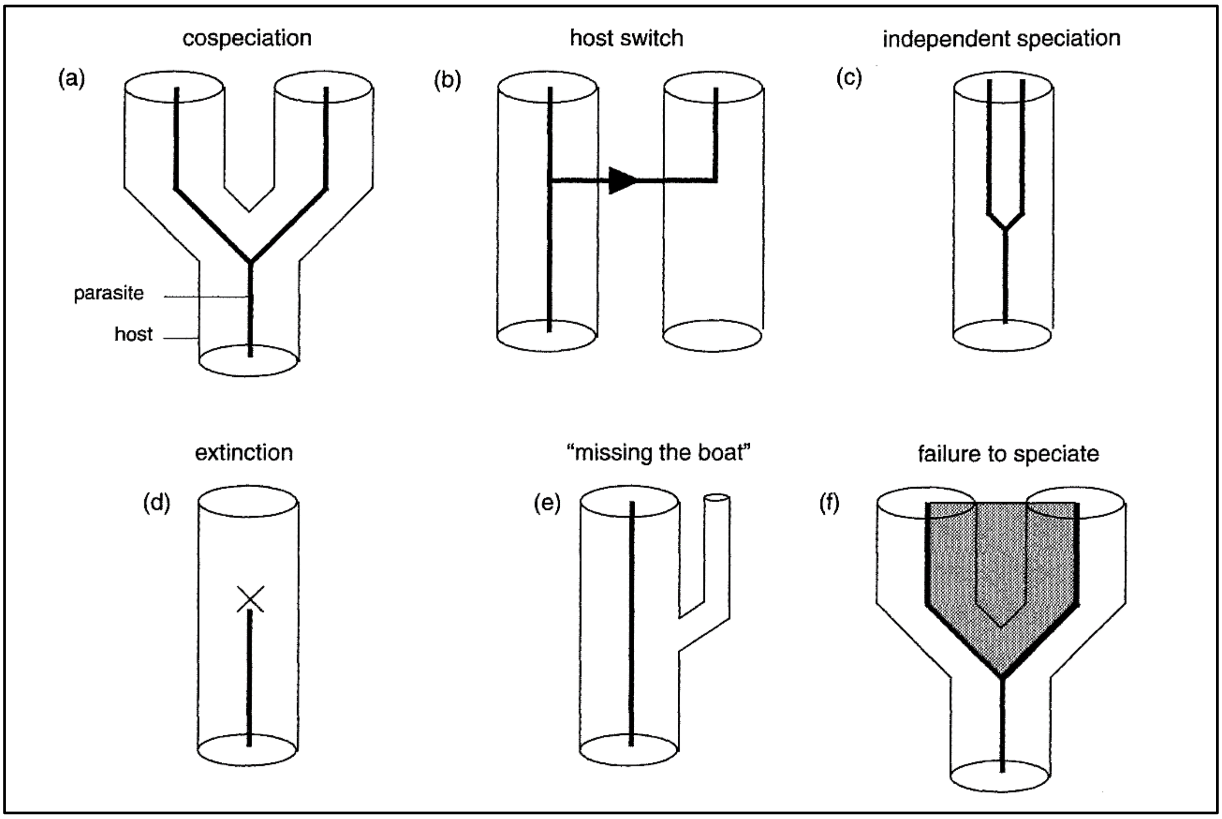

Figure 1. Graphical schemes of six coevolutionary events (Page, 2003)
Host phylogeny is represented by the circular columns and parasite phylogeny by black lines. Independent speciation = intrahost duplication; extinction and "missing the boat" = sorting events.

3.1.2 Cophylogenetic methods for study of host-parasite coevolution

Popularity of cophylogenetics has risen over last few decades and has become a fundamental approach in the investigation of evolutionary trends in host-parasite associations. The studies applying cophylogenetic methods usually employ dual-based approach – combination of distance-based methods and event-based methods (e.g. Desdevises, 2007, Mendlová et al., 2012, Deng et al., 2013, Filipiak et al., 2016, Martínez-Aquino, 2016, Sweet et al., 2016, Zhao et al., 2016, Míguez-Lozano, 2017).

The distance-based methods infer the degree of congruence between host and parasite phylogenies by comparison of genetic distances generated from distance matrices (Johnson et al., 2001, Legendre et al., 2002). The main advantage of this method is that any phylogenetic tree may serve as a template for distance matrices. In the combination with association matrix (i.e. matrix of observed host-parasite associations), some distance-based methods may test significance and contribution of individual host-parasite associations to general cophylogenetic structure by comparing observed association matrices to randomly generated association matrices (Legendre et al., 2002, Desdevises, 2007). The commonly used software for distance-based analyses are following: ParaFit (Legendre et al., 2002), MRCAlink (Schardl et al., 2008), Phylotree (Arnaoudova et al., 2010), and PACo (Balbuena et al., 2013).

The event-based methods implement in the analyses the probability of each of five abovementioned cophylogenetic events. In general, it is assessed by reconciliation of parasite and host trees (Page, 1994, Page & Charleston, 1998), or by character optimization where parasites are considered as host characters (Brooks & McLennan, 1991). The event-based methods aim to reconstruct the most parsimonious coevolutionary history of hosts and parasites, and each incongruence between parasite and host trees is considered as event other than cospeciation. Using fully resolved phylogenetic trees (i.e. without polytomies) is of the high importance, whereas each phylogenetic uncertainty or branch displacement can lead to significantly different results. Moreover, some software allow us to set a penalization cost of each coevolutionary event, thus good estimation of each cost has to be considered and individually evaluated in regards to studied host-parasite system. The estimation of time of divergence of individual lineages also plays important role to differentiate between coevolutionary events and potentially preclude the impossible host switches (Charleston & Page, 2002, Desdevises, 2007, Filipiak, 2016). The commonly used software for event-based analyses are following: TreeFitter (Ronquist, 1995), TreeMap 3.0 (Charleston, 2012), and Jane 4.0 (Conow *et al.*, 2010). List of additional available software for each method was compiled by Martínez-Aquino (2016).

3.1.3 Evolution of host specificity in parasites

Host specificity in parasites has been defined using several criteria; however, in the basic sense, host specificity is expressed by the range of host species which particular parasite species can use during one ontogenetic stage (Poulin, 2007). Generally, two groups of parasites have been recognized on the basis of observed host range: specialists and generalists. While specialist parasite species can infect and live only on/in one host species, generalists use rather wider host range (at least two host species). The host range of generalist may include as congeneric hosts (however, this group of parasites is often termed as intermediate specialists), but phylogenetically distant host taxa as well. Nevertheless, the on-going research unveils that host specificity of numerous parasite species is either under- or overestimated, since reported host specificity is often strongly influenced by sampling size (i.e. number of specimens investigated per host species, numbers of localities investigated for host species, or number of researchers interested in a given parasite group or species and the rate of their research effort (Poulin & Keeney, 2008, Poulin *et al.*, 2019)). As the abundance of parasite species appears to be the most appropriate indicator of the success to infect hosts, Rohde (1980) used this parameter to develop an index for evaluation of the host specificity. At the same time, Euzet and Combes (1980) defined three degrees of host specificity for parasites: (1) strict specificity, where the parasite species can live only on single host species, (2) narrow specificity, where the parasite species may infect phylogenetically close host species (e.g. congeners), and (3) wide specificity, where parasite is not limited to any host species unless they do not share ecological conditions.

To avoid extinction, it was hypothesized that organisms tend to adapt to the most stable resource (Ward, 1992), which in case of host-parasite systems is usually represented by the largest and the most abundant available host species for a given parasite species. Moreover, the host specificity in the parasites is a result of adaptive coevolutionary process (Brooks & McLennan, 1991, Begon *et al.*, 1996), thus over the evolutionary time parasites tend to more specialise to their respective hosts (i.e. Manter's rule, Manter (1955, 1966)). Therefore, we can assume that parasites exhibiting narrow host specificity may use the largest hosts, i.e. host with the highest longevity, which was also previously shown for helminth parasites (Morand *et al.*, 1996, Sasal *et al.*, 1999, Desdevises *et al.*, 2002b, Šimková *et al.*, 2006b).

In addition to basic host specificity (defined as the number of host species used by a given parasite species), Poulin *et al.* (2011) summarized the different views on host specificity. Besides of basic specificity, we can recognize structural, phylogenetic, and geographic specificity. Structural specificity considers ecological contribution of parasite species and is measured by prevalence or abundance of a parasite species in a given host species (Rohde &

Rohde, 2008, Marques *et al.*, 2011). The phylogenetic specificity considers phylogenetic relationships of host species infected by a given parasite species (Humphery-Smith, 1989, Poulin & Mouillot, 2003, Kvach & Sasal, 2010, Archie & Ezenewa, 2011, Cooper *et al.*, 2012). The lastly, geographic host specificity includes the information on geographical distribution of host species used by a given parasite species (Krasnov *et al.*, 2011a,b). By the combination of latter two types of specificities we can compare phylogenetic specificity of given parasite species across several geographical regions. In some regions parasite may exploit large range of hosts, but in another geographical region absence of optimal hosts may be compensated by phylogenetically unrelated host species. To take into account this geographical discrepancy in host specificity Krasnov *et al.* (2011a) proposed term of beta-specificity (or phylobetaspecificity) (Poulin *et al.*, 2011).

Monogeneans are putatively one of the most host-specific parasites (Kearn, 1998, Whittington *et al.*, 2000). According to data presented by Rohde (1979) and also considering host-parasite checklists compiled on different monogenean taxa (e.g. Llewelyn *et al.*, 1984, Gibson *et al.*, 1996, Fletcher *et al.*, 1998, Kohn *et al.*, 1998) majority of monogenean species is restricted to single host species. Such limited host range and high degree of host specificity is the most likely intimately linked to numerousness of adaptations which monogeneans developed to their respective hosts.

3.2 Monogenean parasites as proxies to study host-parasite coevolution

3.2.1 Determinants indicating narrow host specificity in monogeneans

Monogeneans exhibit remarkably high species diversity (over 5,500 species according to Řehulková *et al.*, 2018). As there are mainly the parasites of ectothermic chordates and several invertebrate taxa (i.e. crustaceans and cephalopods) their monoxenous life cycle is restricted to aquatic environment. During development larvae called oncomiracidium (in case of oviparous monogeneans) actively searches for suitable host and subsequently attach to this host. The dispersion of larvae is relatively limited; however, larvae are capable to survive without the host for short period of time (Brooks & McLennan, 1991). In case of viviparous monogeneans (i.e. gyrodactylids), the mode of dispersion is slightly different (see Figure 2 for comparison of life cycle of oviparous and viviparous monogeneans). Since the ciliated stage is absent in their life cycle, they have to rely on the physical contacts of the hosts, otherwise the transmission is rather random and limited only to water currents (Buchmann & Lindenstrøm, 2002, Bakke *et al.*, 2007). After infection of new host, monogeneans actively reach the specific site on or within a host, attach to it, develop into adults (if the larval stage is present in life cycle), and reproduce. The specific site on the host (i.e. microhabitat) may significantly differ in the composition and structure and since microhabitat preference varies among monogenean taxa, they developed different, highly specialised apparatuses, i.e. haptor (=opisthaptor) to attach to the specific microhabitats within hosts (Rohde, 1979).

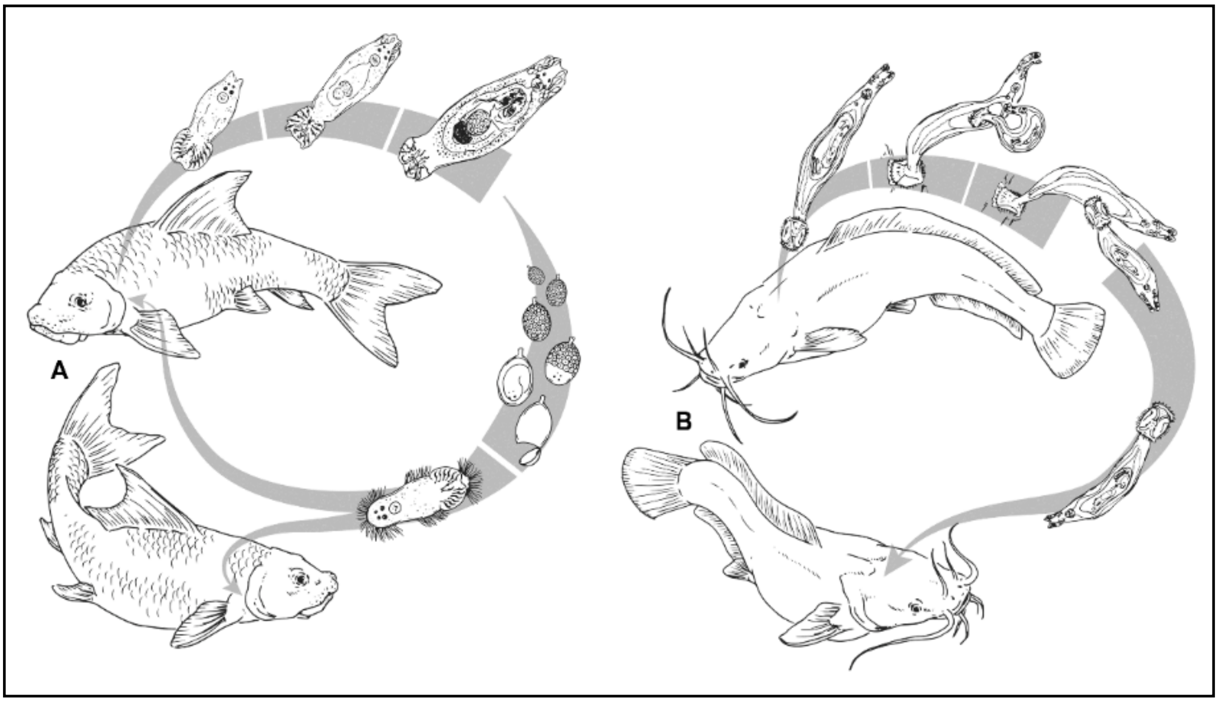

Figure 2. Life cycles in oviparous (left) and viviparous (right) fish monogeneans (Řehulková *et al*., 2018)
A = life cycle of Dactylogyrus *sp.; B = life cycle of* Gyrodactylus *sp.*

3.2.1.1 Dispersal adaptations

To find suitable host, larvae in the case of oviparous monogeneans, and adults in the case of viviparous gyrodactylids, had to develop specific mechanisms for host recognition. However, due to active search, mechanisms of larvae are more important for detection and are rather highly host-specific. Oncomiracidia expose various sensilla (chemo-, photo-, and mechanoreceptors) in different parts of body which may putatively serve for host finding (Tinsley & Owen, 1975, Whittington *et al.*, 1999, and reviewed in Buchmann & Lindenstrøm, 2002). Moreover, the chemical, mechanical or light cues may also influence the egg-hatching (Whittington & Kearn, 2011). Photosensitive hatching strategy evolved independently in the multiple monogenean lineages and usually in the species parasitizing bottom-dwelling fishes, frequently present in the shallow water (e.g. Kearn, 1982, Whittington & Kearn, 1989). When shaded, the cement holding operculum weakens and enables the larva dislodge it and abrupt from the eggs, since larva have only limited time for attaching to host (Kearn, 1975). The exogenous chemical cues have been observed to initiate hatching of the egg, but are important during active search of larvae as well. Kearn (1975) studied the role of the host-specific proteins in monogenean *Entobdella soleae* (van Beneden & Hesse, 1863) important for host recognition. The eggs hatch spontaneously in the absence of host; however, when the host mucus from skin is presented in water, hatching occurs within minutes disregard its common cycle (Kearn 1974, for more examples of chemical cues inducing hatching see Whittington & Kearn, 2011). Kearn (1967) also showed that oncomiracidia of *E. soleae* can select their specific hosts (*Solea solea*, L.) in the environment. He conducted the experiment and suggested that attachment and subsequent deciliation of larvae occurs only after the exposure to its associated host skin mucus, and parasite's response to mucus from non-associated host is only minimal.

Even more complex is the life cycle of polystomatids; monogeneans generally parasitizing aquatic tetrapods. In comparison to previous species which parasites on fish (i.e. strictly aquatic organisms), these parasites develop hatching and dispersion strategies optimised to hemi-aquatic life of their amphibian hosts. In the anurans, adult polystomatids are normally present in the urinary bladder of adults or gill apparatus of tadpoles. However, their dispersion is limited to the aquatic environment, thus it is necessary for them to detect whether their host is at the moment in such environment. As frog reproduction is also limited to the aquatic environment, their polystomatid parasites developed strategy to synchronize life cycle with host reproduction. The initiation of the shedding of eggs by polystomes is presumably controlled by the host's hormonal activity which can be detected by parasite from digested blood (Tinsley 1983, Armstrong *et al.*, 1997). Subsequently, hatching of eggs is either periodic, to assure the presence of suitable hosts (MacDonald & Combes, 1978), or can be stimulated by mechanical cues (Tinsley & Owen, 1975, Kearn, 1986), and changes in osmotic pressure after eggs enter freshwater (Tinsley, 1978). Polystomatids cope with such temporal limitation for reproduction and successful infection of new hosts by retaining multiple eggs within their uterus and deposit them fully developed that immediately hatch (Tinsley 1983). The further postponing of the egg deposition may reach up to ovoviviparity which was recently reported also in polystomatids (Landman *et al.*, 2018); however, viviparity is typical for gyrodactylids, which completely suppressed oviposition and produce fully developed adults inside of parent (Kearn, 1998, Bakke *et al.*, 2007). Since adult monogeneans are unciliated and therefore, movement outside of host is impossible, the direct transfer may occur only during physical contact of their fish hosts (Kearn, 1998, Buchmann & Lindenstrøm, 2002, Bakke *et al.*, 2007).

3.2.1.2 Morphological adaptations

The haptor of monogeneans usually consists of sclerotized anchors, hooks, clamps, connective bars, or suckers. Composition of haptor, i.e. presence of individual sclerotized elements, is highly diverse among monogeneans and is considered as character of high taxonomic importance. Generally, the higher taxa are divided on the basis of haptor structure (following classification proposed by Boeger & Kritsky, 1993), however small nuances in shapes and sizes of individual haptoral elements are used for species determination among congeners (e.g. see Pugachev *et al.* (2009) for differences among *Dactylogyrus*).

The most important element in the haptor of fish monogeneans are usually sclerotized hooks and hook-like structures, or clamps which provide mechanical attachment to the host. Nevertheless, notable exception represents the members of Capsalidae possessing haptor able to generate suction, presumably providing opportunity to parasitize wider host range (Kearn, 1994, reviewed in Whittington, 2004). In contrast to fish monogeneans, haptor of monogeneans parasitizing amphibious tetrapods usually consists of multiple pairs of suckers.

The chemical composition of haptoral sclerites in monogeneans was previously studied, but appears to be still unresolved. Lyons (1966) suggested that sclerites of monogeneans were composed of scleroproteins similar to keratin. Contrastingly, Kayton (1983) argued that sclerites are composed of collagen-like scleroprotein. Further exhaustive research focussed on *Gyrodactylus* von Nordmann, 1832 (Shinn *et al.*, 1995) yielded that individual sclerotized elements of one monogenean species (in their study three *Gyrodactylus* species – *G. salaris* Malmberg, 1957, *G. colemanensis* Mizelle & Kritsky, 1967, and *G. caledoniensis* Shinn, Sommerville & Gibson, 1995) actually differ in the composition and only hammuli are primarily composed of keratin-like protein. The different elemental composition resembling resilin (elastomeric protein common in arthropods) was suggested to be present in the clamps of *Diplozoon paradoxum* von Nordmann, 1832 (Wong *et al.*, 2012). The different structural composition of clamps imply different evolutionary origin of the monogeneans possessing these attachment structure when comparing to hooks of mucus-feeding monogeneans, the structures presumably evolved from suckers (Kearn, 1994).

Nonetheless, due to their thickness and lower plasticity (i.e. lower risk of deformation) sclerotized parts of haptor represent optimal morphological characters for species delineation and therefore, are often prioritized by taxonomists rather than other structures (i.e. internal organs). Moreover, considering that haptor has a functional role it is possible to expect high degree of monogenean adaptation to different types of substrates provided by hosts, i.e. different sites on the given host. Comprehensive research was conducted especially on the monogeneans parasitizing fish. Putatively, monogeneans developed enlarged anchor hooks (=hammuli) after their expansion to the gills (Kearn, 1994). However, the differences in the structure of haptor, i.e. presence of connective bars, number of pairs of anchor hooks, marginal hooks, or additional sclerites, such as squamodiscs, are rather remarkable between different genera of polyonchoineans (see Řehulková *et al.*, 2018 for examples of haptoral sclerites patterns). Due to development of highly specialised attachment organs, congeneric monogeneans exhibit rather high specificity to specific microhabitats. Moreover, due to commonly low population density of gill monogeneans, to facilitate successful mating contacts their distribution is usually limited to only specific site on the gills apparatus (Rohde, 1979, Rohde & Hobbs, 1986, Matějusová *et al.*, 2003). However, Kadlec *et al.* (2003) suggested that reduction of microhabitat may be also linked to interspecific competition between gill monogeneans. As the individual sites provide different kinds of substrates, i.e. gill lamellae differ in shape and size, or different environmental factors, i.e. strength of water flow differs between individual gills arches, presence and/or size of haptoral sclerites of a given monogenean species is linked with the preferred microhabitat provided by host (e.g. Gutiérrez & Martonelli, 1999, Sasal *et al.*, 1999, Šimková *et al.*, 2000, 2002, 2006b, Morand *et al.*, 2002, Huyse & Volckaert, 2002, Jarkovský *et al.*, 2004).

Several studies suggested that shape of haptoral elements is more suitable morphological feature for investigation of phylogenetic relationships between congeners, rather than sclerotized parts of copulatory organs (i.e. vaginal armament and male copulatory organ) which evolutionary rate is rather higher (Pouyaud *et al.*, 2006, Vignon *et al.*, 2011, Mendlová *et al.*, 2012, Mandeng *et al.*, 2015). Moreover, the recent application of geometric morphometric methods, in addition to classic morphological approach, revealed that shape of haptoral anchor hooks of monogeneans is also suitable character for population studies due to their phenotypic plasticity, putatively reflecting the ecology and morphology of hosts (Dmitrieva & Dimitrov, 2002, Olstad *et al.*, 2009, Vignon *et al.*, 2011, Rodríguez-González *et al.*, 2015, Khang *et al.*, 2016, Kmentová *et al.*, 2016).

3.2.2 Monogeneans in host-parasite coevolutionary studies

All abovementioned adaptations are putatively linked with high host specificity of monogeneans. Thus, such host-specific parasites may appear to be suitable for studying host-parasite coevolutionary relationships based on the cophylogenetic approach, as some congruence between host and parasite phylogenies may be hypothesized. However, the studies on the different Monogenea-fish host systems revealed that phylogenetic relationships between these two groups are actually far more complex.

Using cophylogenetic approach Desdevises *et al.* (2002a) investigated degree of congruency between phylogenies of marine fish belonging to Sparidae and their host-specific monogeneans of genus *Lamellodiscus* Johnston & Tiegs, 1922. Whereas *Lamellodiscus* parasitize primarily on representatives of Sparidae (presence on other potential hosts is very rare according to Whitehead *et al.*, 1986), the high degree of congruency between fish and monogenean phylogenies was expected. Nevertheless, their study revealed that cospeciation was almost absent in this host-parasite system and host switching was more common, which was closely associated with the ecology of their sparid hosts. Thus, Desdevises *et al.* (2002a) observed the incongruent host and parasites phylogenies (i.e. phylogenetically close sparid species were not parasitized by phylogenetically proximal *Lamellodiscus* species).

Excessive research was also done on the phylogenetic relationships between viviparous *Gyrodactylus,* which display the widest host range among monogeneans (Harris *et al.*, 2004). Nevertheless, also many *Gyrodactylus* species are highly host-specific (Bakke *et al.*, 2002, 2007). By application dual-based cophylogenetic approach, Huyse and Volckaert (2005) revealed that *Gyrodactylus* species parasitizing the gills originated from host switch and only less host-specific fin *Gyrodactylus* co-speciated with their goby hosts, indicating that in this system cospeciation is not associated with the high host specificity in parasites. Moreover, they showed that host switching of *Gyrodactylus* from *Gasterosteus aculeatus* L. to non-congeneric fish hosts the most likely facilitated adaptive radiation of numerous highly host-specific *Gyrodactylus* species. According to Hahn *et al.* (2015) the cophylogenetic patterns are trackable also on the population level of *Gyrodactylus* parasites of *G. aculeatus*. Their study also supported host switch as a common evolutionary event in the evolutionary history of *Gyrodactylus*.

Contrastingly, *Thaparocleidus* Jain, 1952 represents the genus of strictly host-specific parasites of fish belonging to Pangasiidae (Lim *et al.*, 2001, Pariselle *et al.*, 2006). According to Šimková *et al.* (2013b), intra-host duplication is the main coevolutionary event in this host-parasite system. The similar pattern of coevolution was observed in the system of riverine fish belonging to Cichlidae and their host-specific gill monogeneans – *Cichlidogyrus* Paperna, 1960 and *Scutogyrus* Pariselle & Euzet, 1995 (Mendlová *et al.*, 2012). Šimková *et al.* (2004) tackled the question about the main coevolutionary event driving speciation of *Dactylogyrus* parasitizing central European Cyprinoidei, and suggested that it is intra-host duplication, congruently with the abovementioned host-parasite systems as the main coevolutionary event in the system including riverine fish and their host-specific monogeneans.

3.3 Diversity and host specificity of *Dactylogyrus* parasites

3.3.1 Overall species diversity of *Dactylogyrus*

Dactylogyrus is the monogenean genus with currently the highest known species diversity among Platyhelminthes (more than 900 species according to last checklist compiled by Gibson *et al.*, 1996). Majority of species belonging to this genus are gill parasites; however, a few exceptions represent *Dactylogyrus* species parasitizing in the nasal cavity (e.g. *D. yinwenyingae* Gussev, 1962, *D. olfactorius* Lari, Adams, Cone, Goater & Pyle, 2016). Biogeographical distribution of *Dactylogyrus* concurs with the distribution of their cyprinoid fish hosts, as their occurrence in native cyprinoids was documented in Africa, Asia, North America and Europe. Even thought, *Dactylogyrus* are parasites of fishes of high scientific interest (Cyprinoidei), their diversity is still underexplored. A total of 40 new species were described in the last two decades (Jalali *et al.*, 2000, Cloutman & Rogers, 2005, Řehulková & Gelnar, 2005, Cloutman, 2006, 2009, 2011, Musilová *et al.*, 2009, Crafford *et al.*, 2012, Modu *et al.*, 2012, Aydoğdu *et al.*, 2015, Lari *et al.*, 2016, Nitta & Nagasawa, 2016, 2017, Rahmouni *et al.*, 2017, Wangchu *et al.*, 2017, Mashego & Katlou, 2018, Paper I and Paper VI of this doctoral thesis). Moreover, many new recently discovered *Dactylogyrus* species are undescribed yet (e.g. see Papers IV, V), and recent application of species delimitation methods is gradually uncovering cryptic diversity in *Dactylogyrus* (Papers III, V).

Each cyprinoid species may potentially serve as a host for at least one *Dactylogyrus* species and divergent host lineages are usually parasitized by highly host-specific *Dactylogyrus* species (see Ergens, 1970 and Paper I for parasites of *Pachychilon pictum* (Heckel & Kner, 1858) and *Aulopyge huegelii* Heckel, 1843 respectively). Therefore, the remarkable species diversity of *Dactylogyrus* might be explained as reflection of the diversity of their cyprinoid hosts. Nonetheless, it is not uncommon that cyprinoid host harbours species-rich *Dactylogyrus* communities (Šimková *et al.*, 2000, 2004, Paper III). Especially the host species with wide distribution range in Europe, such as *Squalius cephalus* L., *Rutilus rutilus* L., and *Cyprinus carpio* L. were documented to be parasitized by up to ten *Dactylogyrus* species (Šimková *et al.*, 2000, Seifertová *et al.*, 2008, Molnár, 2012 and references wherein). High number of *Dactylogyrus* species was reported also on African *Labeo coubie* Rüppell, 1832 (11 species) and North-American *Luxilus cornutus* (Mitchill, 1817) (12 species) whose distribution is rather widespread in respective regions, and covers several major drainages (Reid, 1985, Page & Burr, 1991, Hoffman, 1999, Musilová *et al.*, 2009). This is in the line with Gregory (1990) hypothesizing that hosts with larger area of distribution harbour species richer parasite communities due to broader contact zones with other phylogenetically close hosts. Such contact zones putatively facilitate host switching of parasites between hosts. However, in the case of host specific monogeneans, wide distribution range of given host species may promote vicariant speciation in parasites due to their faster evolutionary rate (Poulin, 2007). Therefore, we can assume that fish species with limited distribution range (i.e. endemics) will be, in general, parasitized by species poorer *Dactylogyrus* communities.

Supporting such claim, in the southern European Peninsulas where distribution of the cyprinoid fish hosts is usually restricted to small regions (e.g. single river basin), and freshwater fauna consists of numerous highly endemic species (Kottelat & Freyhof, 2007), cyprinoids are parasitized in average by two *Dactylogyrus* species (Dupont & Lambert, 1986, el Gharbi *et al.*, 1992, Galli *et al.*, 2002, 2007, Stojanovski *et al.*, 2004, 2005, 2012, Paper III, Paper V). Reports of species rich *Dactylogyrus* communities from southern European cyprinoids are rare and maximum five *Dactylogyrus* species were found on a single endemic host species (el Gharbi *et al.*, 1992).

Multiple abiotic and biotic factors may influence species composition of *Dactylogyrus* communities. For example, presence of *Dactylogyrus* species depends on season, and especially on water temperature (Simón-Vicente, 1981, González-Lanza & Alvarez-Pellitero, 1982, Lux, 1990, Šimková *et al.*, 2001b), therefore, shifts in the species composition of parasite communities may be observed during the year. The host size has also impact on the composition of the *Dactylogyrus* communities. Larger fish provide more space for parasites and therefore, may potentially harbour species richer parasite communities. Positive correlation between host body size and species richness of *Dactylogyrus* communities was revealed by Šimková *et al.* (2001a). Moreover, hybridization between phylogenetically close host species may promote host switching of the parasites and produces distortions in expected communities, i.e. widen species richness of parasite communities (Šimková *et al.*, 2013a, Krasnovyd *et al.*, 2017).

3.3.2 Host specificity of *Dactylogyrus* parasites

As mentioned above, *Dactylogyrus* are almost strictly gill-specific parasites of cyprinoid fishes. Nevertheless, larval or subadult stages may be found also on the fins or skin from where they migrate to gill chamber, attach to gill lamellae and mature. Haptor of the *Dactylogyrus* parasites comprises of one pair of anchor hooks, usually one dorsal and one ventral connective bar of various shapes (ventral connective bar may be absent) and seven pairs of marginal hooks (see Pugachev *et al.*, 2009 for morphotypes of haptoral sclerotized elements, basic scheme is presented in Figure 3). Position of *Dactylogyrus* species on the gill apparatus is putatively species specific (Šimková *et al.*, 2000, Turgut *et al.*, 2006) and preference of specific microhabitat is associated with the shape and size of the haptoral anchor hooks, i.e. species inhabiting overlapping microhabitats share morphologically similar haptoral sclerites (Šimková *et al.*, 2000, reviewed in Šimková & Morand, 2008). This hypothesis was tested also by Jarkovský *et al.* (2004), who revealed that host-specific *Dactylogyrus* species in their infracommunities share more similarities in haptoral morphometrics than species in infracommunities of generalists. The haptor morphometrics and presence of specific sclerites apparently play important role in the host specificity of *Dactylogyrus*. Some generalist species (e.g. *D. sphyrna* Linstow, 1878, *Dactylogyrus vastator* Nybelin, 1924, and *Dactylogyrus vistulae* Prost, 1957) have not developed haptoral ventral connective bar. As this morphological feature is present in numerous *Dactylogyrus* species, it was hypothesized (Paper VII) that secondary loss of ventral connective bar allows parasite to infect wider range of host. Concurrently, Šimková *et al.* (2006b) hypothesized that attachment organs with large sized sclerites promote colonization of several phylogenetically distant host species.

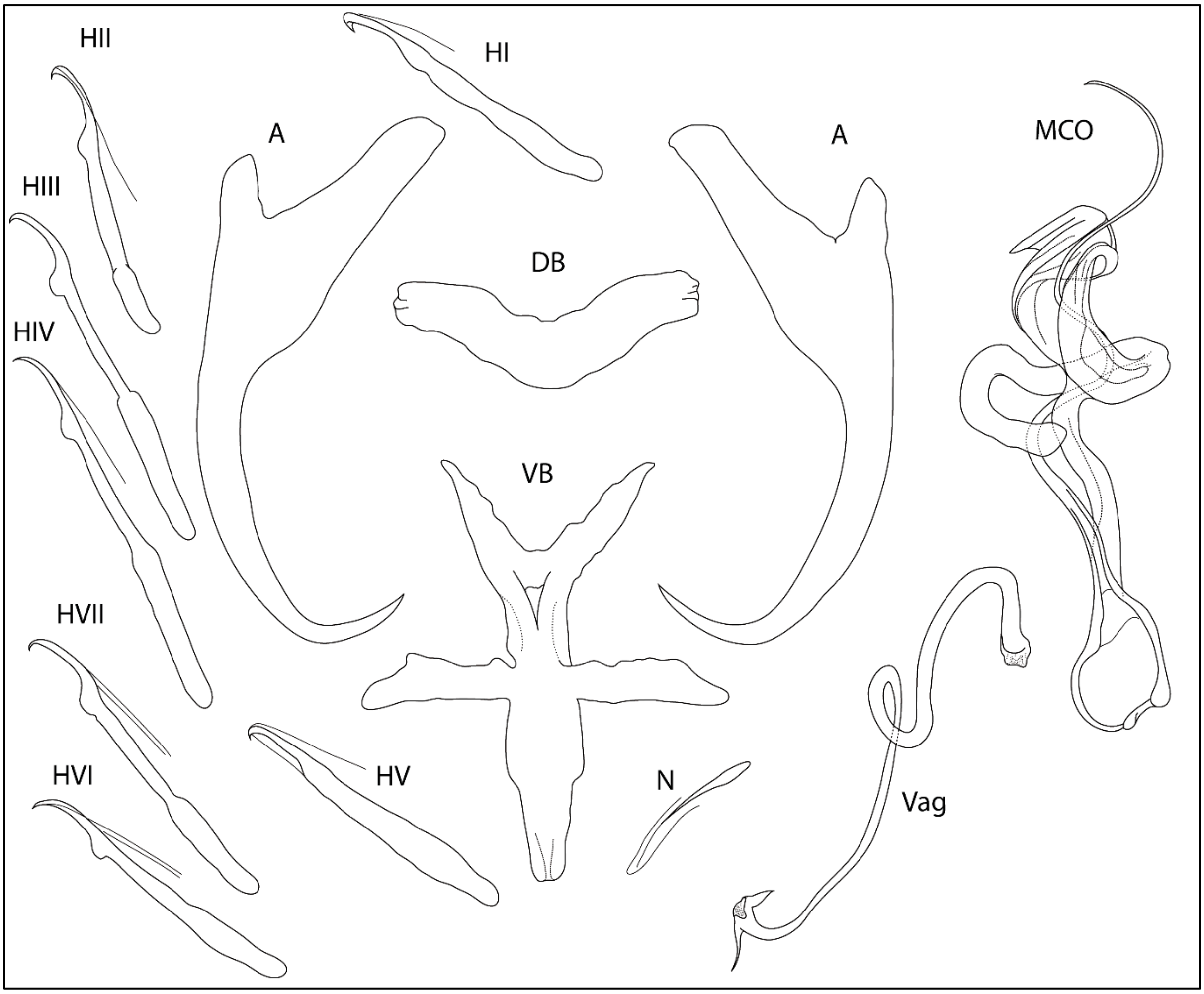

Figure 3. Schematics of basic of composition of haptoral sclerites and sclerotized parts of copulatory organs in *Dactylogyrus* (from Paper I)
HI-VII = 7 pairs of marginal hooks; A = anchor hooks; DB = dorsal connective bar; VB = ventral connective bar; N = needle, MCO = male copulatory organ; Vag = vaginal armament

Dactylogyrus parasites generally exhibit high degree of host specificity (el Gharbi *et al.*, 1992, 1994, Lambert & el Gharbi, 1995, Šimková *et al.*, 2004, 2006b, Šimková & Morand, 2008, Paper I, Paper VI); however, dispersal capabilities of larval stages putatively promote colonization of new hosts. On the basis of number of host species and their phylogenetical proximity, Desdevises *et al.* (2002b) classified degrees of host specificity based on the index of non-specificity and applied this index for monogeneans of the genus *Lamellodiscus*. Later, Šimková *et al.* (2006b) adapted this classification and defined five classes of host specificity for *Dactylogyrus* species: (1) the strict specialists parasitizing single host species, (2) intermediate specialists parasitizing congeneric cyprinid species (following old classification of cyprinids), (3) intermediate generalist parasitizing non-congeneric, phylogenetically proximal, cyprinid species, (4) generalists parasitizing cyprinid species from different subfamilies, and (5) real generalists parasitizing any, phylogenetically unrelated, host species.

3.4 Phylogeny and biogeography of cyprinoids

3.4.1 Phylogeny of Cyprinoidei

Former family Cyprinidae recently underwent significant taxonomical revision. Tan & Armbruster (2018) and Schönhuth *et al.* (2018) elevated the Cyprinidae to the suborder level i.e. Cyprinoidei which includes all members of Cyprinidae *sensu lato,* and genera *Psilorhynchus* McClelland, 1833 and *Paedocypris* Kottelat, Britz, Tan & Witte, 2006. Cyprinidae *sensu lato* initially included eleven subfamilies, majority of which were elevated to level of families and currently Cyprinoidei consists of 12 families: Acheilognathidae, Cyprinidae *sensu stricto,* Danionidae, Gobionidae, Leptobarbidae, Leuciscidae, Paedocypridae, Psilorhynchidae, Sundadanionidae, Tanichthyidae, Tincidae, and Xenocyprididae. In general, there are no exclusive synapomorphies for Cyprinoidei except presence of pharyngeal teeth in one to three rows which are used to grind up the food against a masticatory pad on the basioccipital process of the cranium (Chu, 1935, Nelson, 2006). A mixture of traits are commonly used to characterize the clades within cyprinoids, such as presence of barbels (pair of tegumental exercences with tactile and gustative receptors, Howes, 1991) or spine-like rays in the dorsal fin (Nelson, 2006).

Phylogenetic affinity of *Psilorhynchus* was uncertain for a long period of time, even thought, monophyly of this clade is suggested by numerous morphological synapomorphies (Conway, 2011). This genus was on the basis of morphology previously classified either within cobitoids or cyprinoids, and only subsequently was defined as monogeneric family Psilorhynchidae (Conway *et al.*, 2010, Conway, 2011). Nevertheless, recent molecular phylogenetic studies revealed sister position of Psilorhynchidae to Cyprinidae *sensu stricto,* thus rendering Cyprinidae *sensu lato* paraphyletic (Hirt *et al.*, 2017, Schönhuth *et al.*, 2018). The genus *Paedocypris* was erected only recently, and comprises of miniature fishes, including supposedly the smallest vertebrates in the world (Kottelat *et al.*, 2006). Formerly classified as genus belonging to Danionidae (Rüber *et al.*, 2007, Tang *et al.*, 2010) the *Paedocypris* was promoted by the molecular phylogenetics to family level (Stout *et al.*, 2016, Hirt *et al.*, 2017, Schönhuth *et al.*, 2018) and is easily distinguishable among cyprinoids by numerous autapomorphic characters (see Kottelat *et al.*, 2006). Nonetheless, genera *Leptobarbus* Bleeker, 1860, *Sundadanio* Kottelat & White, 1999, and *Tanichthys* Lin, 1932 share synapomorfic trait (*Y*-shaped ligament connecting kinethmoid and ethmoid), all three were previously also included in the Danionidae which all other representatives have this ligament absent (Kottelat & Witte, 1999, Liao & Kullander, 2012). Further molecular phylogenetic studies revealed divergent position of three abovementioned genera to danionids (Chen & Mayden 2009; Mayden & Chen 2010; Stout *et al.* 2016; Hirt *et al.* 2017 Schönhuth *et al.*, 2018) leading to erection of separated families Leptobarbidae, Sundadanionidae and Tanychthyidae (Tan & Armbruster, 2018). In the peri-Mediterranean region the majority of the endemic cyprinoid species belong to two of the most species rich families: Cyprinidae and Leuciscidae.

Following the recent classification, Cyprinidae *sensu stricto* encompasses more than 1300 species (Fricke *et al.*, 2019) belonging to eleven morphologically diverse subfamilies (formerly tribes, as defined by Yang *et al.*, 2015) with the unequal distribution across Africa and Eurasia. Cyprinids exhibit extremely varying levels of polyploidy, resulting from ancestral hybridization and subsequent polyploidization, which recently played important role in the classification of the subfamilies within Cyprinidae (Yang *et al.*, 2015). Noteworthy is subfamily Barbinae which encompasses majority of endemic European cyprinid species (Kottelat & Freyhof, 2007). Barbinae formerly included also diploid African *Enteromius* Cope, 1867 (i.e. ‘small African barbs’) and many other genera currently included into Torinae (e.g. *Pterocapoeta* Günther, 1902 and *Carasobarbus* Karaman, 1971) and Smiliogastrinae (*Barboides* Brüning, 1929 and *Dawkinsia* Pethyiagoda, Meegaskumbura & Maduwage, 2012). Recently, Barbinae includes only monotypic European *Aulopyge* Heckel, 1841, Eurasian tetraploid and African diploid *Barbus* Cuvier & Cloquet, 1816 (Hayes & Armbruster, 2017), *Luciobarbus* Heckel, 1843 with the distribution range across Eurasia and Africa, and Asian *Capoeta* Valenciennes, 1773, *Cyprinion* Heckel, 1843, *Scaphiodonichthys* Vinciguerra, 1890, and *Semiplotus* Bleeker, 1859. Five dispersion events were proposed to explain the present biogeographical distribution of cyprinid lineages, i.e. subfamilies (see revision in Yang *et al.*, 2015).

In comparison to Cyprinidae, Leuciscidae are distributed primarily in Eurasia and North America where they represent the most common cyprinoid assemblages (Cavender & Coburn, 1992, Kottelat & Freyhof, 2007). For a long period of time the phylogenetic relationships between the clades within leuciscids were not established, since most of the species are morphologically similar (Howes, 1991). Formerly leuciscids (at that time Leuciscinae) were divided into several subclades of which two major were Leuciscinae (former Leuciscini) and Phoxininae (former Phoxini) (Cavender & Coburn, 1992) The former one included primarily Eurasian genera and single Nearctic species (*Notemigonus crysoleucas* (Mitchill, 1814)), and the latter primarily all native North American genera (except abovementioned monotypic *Notemigonus*) and some Eurasian genera (Cavender & Coburn, 1992, Kottelat & Freyhof, 2007, Imoto *et al.*, 2013, Schönhuth *et al.*, 2018). While the previous classification of Leuciscinae remained after recent taxonomic reevaluation almost intact, former Phoxininae were due to the paraphyletic status divided into five subfamilies, each corresponding to major clades proposed in recent studies: Laviniinae for the Western clade, Phoxininae for only Eurasian species, Plagopterinae for Creek Chub-Plagopterins, Pogonichthyinae for OPM clade (‘open posterior myodome’), and Pseudaspininae for the Far East clade (Bufalino & Mayden, 2010, Schönhuth *et al.*, 2012, 2018, Imoto *et al.*, 2013). Currently Phoxininae is monogeneric and the distribution range of *Phoxinus* Rafinesque, 1820 covers only western part of Eurasia (Kottelat & Freyhof, 2007, Tan & Armbruster, 2018). The other newly erected subfamilies encompass species from the Far East (Pseudaspininae), and North America (Laviniinae, Plagopterinae and Pogonichthyinae).

The general dispersal hypothesis assumed that leuciscids originated in the Asia, they dispersed into Europe (Perea *et al.*, 2010) and colonized Nearctic via Beringia land bridge (Cavender, 1991). However, according to robust molecular phylogenies, Imoto *et al.* (2013) proposed that North American leuciscids are of European origin and during two dispersion events colonized North America via land bridges (e.g. Thulean Bridge, McKenna (1983), Tiffney (1985)) and only subsequently colonized Far East via Beringia.

3.4.2 Species diversity and distribution of cyprinoids in the peri-Mediterranean area

The peri-Mediterranean area stretches over three continents and encompasses three geopolitical regions: Euro-Mediterranean (i.e. southern Europe), Afro-Mediterranean (i.e. Maghreb), and Middle East (i.e. Levant). The majority of species of peri-Mediterranean freshwater fish fauna belong to Cyprinoidei, more specifically to Cyprinidae and Leuciscidae (Bănărescu & Coad, 1991, Kottelat & Freyhof, 2007). Both families include several endemic genera and high number of endemic species (list of endemic genera is presented in Table 1). While Cyprinidae are represented in peri-Mediterranean by endemic species belonging to eight genera; *Aulopyge, Barbus, Capoeta, Carasobarbus, Garra* Hamilton, 1822, *Labeobarbus* Rüppell, 1835, *Luciobarbus,* and *Pterocapoeta* (only monotypic *Aulopyge* and *Pterocapoeta* are endemic to peri-Mediterranean), Leuciscidae are currently represented by endemic species belonging to 25 genera (of which 18 are endemic to peri-Mediterranean). Some of these genera exhibit high intergeneric morphological similarity and/or close phylogenetic relationships, i.e. *Barbus* and *Luciobarbus; Telestes* Bonaparte, 1837 and *Squalius* Bonaparte, 1837*;* or recently erected genera from *Chondrostoma s.l. – Parachondrostoma* Robalo, Almada, Levy & Doadrio, 2007 and *Pseudochondrostoma* Robalo, Almada, Levy & Doadrio, 2007 (Kottelat & Freyhof, 2007, Robalo *et al.*, 2007). Overall, peri-Mediterranean area is divided into 12 well-defined and three uncertainly defined ichthyogeographical districts (*sensu* Bianco (1990)), each delineated on the basis of recent distribution of primary freshwater fishes, in combination with fossil records and paleogeographical data.

Geographically the Euro-Mediterranean region encompasses three topologically different peninsulas – Apennine, Balkan, and Iberian. In comparison to central and northern Europe, where the cyprinoid fauna is relatively uniform and includes only several species with the wide distribution range, the faunas of southern European Peninsulas harbour remarkable cyprinoid diversity, extremely rich in endemic species (Reyjol *et al.*, 2007, Kottelat & Freyhof, 2007). However, distribution of individual genera is rather unequal across peninsulas. While the Iberian Peninsula harbours almost exclusively endemic cyprinoid species (except *Squalius laietanus* Doadrio, Kottelat & Sostoa, 2007, *Phoxinus bigerri* Kottelat, 2007 and *Barbus meridionalis* Risso, 1827), the number of currently recognized genera is lower than in the Balkans. The only genera with the distribution across all three southern European peninsulas are following: *Barbus*, *Phoxinus* and *Squalius* (Kottelat & Freyhof, 2007).

Due to the topology, historical formation, and multiple rearrangements of the large Lake systems, the Balkan Peninsula is currently one of the most important hotspots of European biodiversity, harbouring approximately 59% of all European cyprinoid species (Sušnik *et al.*, 2007, Abell *et al.*, 2008, Albrecht & Wilke, 2008, Schultheiss *et al.*, 2008, Oikonomou *et al.*, 2014). Especially Dessaretes lake system played the important role in the speciation of cyprinoids in the Balkans. This large connection of water bodies originated in Pliocene and covered area of all present Great Lakes of the Balkan Peninsula; i.e. Ohrid Lake (located on the border of Albania and North Macedonia), Prespa Lake (Albania, Greece and North Macedonia), Mikri Prespa Lake (Albania and Greece) and Maliq Lake (Albania, evaporated during World War II) (Sušnik *et al.*, 2007, Abell *et al.*, 2008, Albrecht & Wilke, 2008, Schultheiss *et al.*, 2008, Bordon *et al.*, 2009, Wagner & Wilke, 2011). Later, after the closing of Korca depression and connections between Paratethys and Dessaretes, the water level gradually decreased, promoting allopatric speciation in the freshwater fauna, and leading to recent rich species diversity in the Great Lakes (Steininger & Rögl, 1984, Albrecht & Wilke, 2008). Nevertheless, number of endemic species and degree of endemism resulting from the split of this Lake system is often overestimated, since past underground hydrological connections promoted faunal sharing of the lakes with the neighbouring drainage, rather than having completely distinct endemic faunas (Albrecht *et al.*, 2008). The highest degree of endemism was reported from lakes Ohrid and Prespa with more than 50% of native fish species endemic for each lake (Stanković, 1960, Crivelli *et al.*, 1997, Albrecht & Wilke, 2008). However, later re-evaluation of the distribution range of several cyprinoid species revealed that some formerly considered as endemic species from Lake Ohrid (e.g. *Pelasgus minutus* (Karaman, 1924), *Rutilus karamani* Fowler, 1977), are present also in the Lake Skadar and River Drini (see Kottelat & Freyhof (2007) for complete distribution range). Therefore, the similar pattern of distribution may be expected for other potential endemics after more thorough surveying. Currently the freshwater fauna of Balkans encompasses endemic cyprinoid species belonging to 17 genera. The most speciose genera in the Balkans are, *Barbus* and *Squalius*, encompassing 11 and 14 endemic species respectively (Kottelat & Freyhof, 2007). In contrast to two aforementioned genera, whose species can be found also in other geographic regions, many cyprinoid genera are present only in the Balkans. Examples are monotypic *Aulopyge* (*A. huegelii*) which distribution is limited to the Dinaric Karst Rivers, Lakes of Croatia, and Bosnia and Herzegovina, or *Pachychilon* Steindachner, 1882 and *Tropidophoxinellus* Stephanidis, 1974, each with two species (Vuković & Ivanović, 1971, Mrakovčić *et al.*, 1990, Kottelat & Freyhof, 2007).

Contrastingly to the species-rich Balkan Peninsula, the Apennine Peninsula harbours only several endemic cyprinoid species, belonging to eight genera, from which one genus (*Protochondrostoma* Robalo, Almada, Levy & Doadrio, 2007) is endemic (Bianco, 1995, Kottelat & Freyhof, 2007). Moreover, according to the molecular data, all endemic species are of more recent origin, the most probably due to fact that large part of the Peninsula was below sea level during most of the Miocene era (Steiniger & Rögl, 1984). The Apennine Peninsula is characterised by two main ichthyogeographical districts; Padano-Venetian, and Tuscano-Latium (Bianco, 1990, 1995). The Tuscano-Latium district corresponds to the distribution of endemic *Squalius lucumonis* (Bianco, 1983) and almost exclusively encompasses endemic species (except *Tinca tinca* L.) (see Kottelat & Freyhof (2007) for the distribution range). On the other hand, Padano-Venetian district covers region ranging from River Vomano, in central Italy, to River Krka, in Dalmatia, and basically corresponds to the drainage of River Po during the Last Glacial Maximum when the sea level drastically regressed (Pielou, 1979, Bianco, 1990, Waelbroeck *et al.*, 2002). It was hypothesized, that drop of sea level and subsequent expansion of Po basin connected the currently isolated Italian and Balkan river systems (Waelbroeck *et al.*, 2002, Stefani *et al.*, 2004), which would explain the fact that cyprinoid fauna shows no or very low molecular divergence between species living on the both sides of the Adriatic Sea, regionally corresponding to Padano-Venetian district (Buj *et al.*, 2010, Perea *et al.*, 2010, Geiger *et al.*, 2014).

Cyprinoid fauna of Iberian Peninsula consists almost exclusively of endemic species, generally each with limited distribution range, rarely overlapping with the distribution range of other congeneric cyprinoids (Doadrio *et al.*, 1990, Kottelat & Freyhof, 2007, Gante *et al.*, 2015). Following recent classification which splits *Chondrostoma* into six monophyletic genera (Robalo *et al.*, 2007), Leucscidae are represented in the Iberia by eight genera. Similarly to the Balkans, the Cyprinidae are represented by *Barbus* and *Luciobarbus* as well; however, the *Luciobarbus* is much more diversified in comparison to *Barbus*, i.e. *Barbus* in Iberia is represented by two species, of which only *B. haasi* Mertens, 1925 is endemic. The second one, *B. meridionalis* is native to the southern France; however, its distribution range reaches up to the north-eastern Iberian drainages where overlaps with the distribution range of *B. haasi* and these two species hybridize (Machordom *et al.*, 1990, Kottelat & Freyhof, 2007). Regarding Leuciscidae, the most endemic species belong to the genera *Squalius* and genera erected from *Chondrostoma sensu lato* (i.e. *Achondrostoma* Robalo, Almada, Levy & Doadrio, 2007, *Iberochondrostoma* Robalo, Almada, Levy & Doadrio, 2007, *Parachondrostoma* and *Pseudochondrostoma*) (Kottelat & Freyhof, 2007, Robalo *et al.*, 2007, Schönhuth *et al.*, 2018). *Phoxinus* was formerly represented in the Iberia by single species *P. bigerri*; however, Corral-Lou *et al.* (2019) proposed the occurrence of three *Phoxinus* species in the northern Iberia based on phylogenetic and phylogeographic analyses using multi-locus genomic data. Apparently, the classification of the *Phoxinus* in the Iberia is far more complex, and similarly as in the Balkans (Palandačić *et al.*, 2015), this genus encompasses complex of multiple morphologically cryptic species. The high degree of endemism in the Iberia is the most likely linked with the geographical isolation of this region. The main routes promoting dispersion into and from this peninsula were through the north-east from the Europe and via the elevated land bridge in the south from the North-west Africa. However, after reopening of the strait of Gibraltar and elevation of Pyrenees, the possibilities for dispersion were limited, rendering the species isolated, and promoting allopatric speciation (Hsü *et al.*, 1973, Rosenbaum *et al.*, 2001). Moreover, the mountainous topology of the Iberian Peninsula provided multiple refuge during glacial periods and promoted further adaptive radiation of the species (Gante *et al.*, 2011, Hewitt, 2011).

Completely different is the situation in the Afro-Mediterranean region (i.e. Maghreb), where the cyprinoid fauna consists exclusively of cyprinid species (Winfield & Nelson, 1991, Tsigenopoulos *et al.*, 2003, Yang & Mayden, 2010, Touil *et al.*, 2019). After the recent classification, two subfamilies are recognized in the Afro-Mediterranean: hexaploid, large scaled Torinae (genera *Carasobarbus, Labeobarbus,* and *Pterocapoeta*), and tetraploid Barbinae (only genus *Luciobarbus*) (see tribes in Yang *et al.*, 2015). Interestingly, while the phylogenetic relationships between *Barbus* and *Luciobarbus* is fully resolved and both genera are distinguishable on the basis of several autapomorphies (e.g. number of pharyngeal teeth), the *Luciobarbus* does not form monophyletic group (Bănărescu and Bogutskaya, 2003, Tsigenopoulos & Berrebi, 2000, Tsigenoipoulos *et al.*, 2003, Levin *et al.*, 2012, Yang *et al.*, 2015). According to the molecular phylogeny, the position of Middle-Eastern *Capoeta* is nested within *Luciobarbus.* Furthermore, the North African *Luciobarbus* are in paraphyly, as endemic species *Luciobarbus setivimensis* (Valenciennes, 1842) and *Luciobarbus guercifensis* Doadrio, Perea & Yahyahoui, 2016 are phylogenetically related to the Iberian *Luciobarbus* (Yang *et al.*, 2015, Doadrio *et al.*, 2016, Touil *et al.*, 2019). The phylogenetic displacement of two abovementioned species may be explained by the proposed Betic-Kabilian plate connecting North Africa with Iberia by Machordom and Doadrio (2001), which provided opportunity for mixing fauna between these two regions.

Gradual closing of Tethys which took a place in Middle East during later Miocene functioned as important center of evolution for euryhaline fauna (Por & Dimentman, 1985). However, the speciation of the freshwater fauna was historically centred in the Mesopotamian Basin, where before the Pliocene orogenesis Proto-Eupfhrates River maintained connection between Black and Caspian Seas and allowed mixture of African and Asian species (Por & Dimentman, 1985, Por, 1989). Therefore, the Middle East is considered to be a major biogeographical crossroad and currently we can found in the local fauna Asian (e.g. *Garra* or *Schizothorax* Heckel, 1838) and African (i.e. *Luciobarbus* and *Carasobarbus*) elements (Durand *et al.*, 2002, Yang *et al.*, 2015). Nevertheless, the Middle East also comprises numerous endemic species and even some endemic genera with distribution only in this region (e.g. *Acanthobrama* Heckel, 1843 or *Capoeta*). In general, the diversity of cyprinoids is not well documented in the Middle East and molecular data on fish are scarce (Çiçek *et al.*, 2015, Esmaeili *et al.*, 2017, 2018). However, species of both families, Cyprinidae and Leuciscidae, are present in this region. The most speciose genera are *Capoeta* (Cyprinidae) and *Pseudophoxinus* Bleeker, 1860 (Leuciscidae), both with about 30 species. While *Capoeta* is, as mentioned above, phylogenetically related to the peri-Mediterranean *Luciobarbus* (Yang *et al.*, 2015), *Pseudophoxinus* is phylogenetically close to European *Telestes* (Perea *et al.*, 2010). Other endemic genera (e.g. *Arabibarbus* Borkenhagen, 2014 or *Leucalburnus* Berg, 1916) include only few species, or are even monotypic (Bogutskaya, 1997, Borkenhagen, 2014, Esmaeili *et al.*, 2018).

Table 1. List of endemic cyprinoid genera in the peri-Mediterranean with information on distribution

Family	Genus	N	Distribution
Cyprinidae	***Aulopyge*** Heckel, 1841	1	BP
	Barbus Cuvier & Cloquet, 1816	16	Euro-M, Middle-East
	Capoeta Valenciennes, 1773	7	Middle-East
	Carasobarbus Karaman, 1971	5	Afro-M
	Garra Hamilton, 1822	1	Middle-East
	Labeobarbus Rüppell, 1835	1	Afro-M
	Luciobarbus Heckel, 1843	24	peri-M
	Pterocapoeta Günther, 1902	1	Afro-M
Leuciscidae	***Acanthobrama*** Heckel, 1843	7	Middle-East
	Achondrostoma Robalo, Almada, Levy & Doadrio, 2007	3	IP
	Alburnoides Jeitteles, 1861	7	BP
	Alburnus Rafinesque, 1820	19	BP, AP, and Middle-East
	Anaecypris Collares-Pereira, 1983	1	IP
	Chondrostoma Agassiz, 1832	13	BP
	Delminichthys Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig, 2006	4	BP
	Iberochondrostoma Robalo, Almada, Levy & Doadrio, 2007	4	IP
	Iberocypris Doadrio, 1980	2	IP
	Ladigesocypris Karaman, 1972	2	BP, Middle-East
	Leucalburnus (Berg, 1910)	1	Middle-East
	Leuciscus Cuvier, 1816	1	Euro-M
	Pachychilon Steindachner, 1882	2	BP
	Parachondrostoma Robalo, Almada, Levy & Doadrio, 2007	4	IP
	Pelasgus Kottelat & Freyhof, 2007	7	BP
	Petroleuciscus Bogutskaya, 2002	2	BP, Middle-East
	Phoxinellus Heckel, 1843	3	BP
	Phoxinus Rafinesque, 1820	4	Euro-M, Middle-East
	Protochondrostoma Robalo, Almada, Levy & Doadrio, 2007	1	AP
	Pseudochondrostoma Robalo, Almada, Levy & Doadrio, 2007	3	IP
	Pseudophoxinus Bleeker, 1860	24	BP. Middle-East, Afro-M
	Rutilus Rafinesque, 1820	9	BP, AP
	Scardinius Bonaparte, 1837	7	BP
	Squalius Bonaparte, 1837	27	Euro-M, Middle-East
	Telestes Bonaparte, 1837	13	BP, AP
	Tropidophoxinellus Stephanidis, 1974	2	BP
	Vimba Fitzinger, 1873	1	Middle-East

Genera in bold are endemic to peri-Mediterranean; N = number of endemic species in peri-Mediterranean from given genera; Distribution = distribution range of species of given genus; BP = Balkan Peninsula, AP = Apennine Peninsula IP = Iberian Peninsula, Afro-M = Afro-Mediterranean; Euro-M = Euro-Mediterranean; peri-M = whole peri-Mediterranean region.

3.4.3 Phylogeography of Euro- and Afro-Mediterranean cyprinoids

Since cyprinoids are primarily freshwater fishes their dispersion capabilities are highly limited. The single exception is *Tribolodon* Sauvage, 1883 which secondary developed broad osmoregulation capabilities and tolerance to saline (brackish) environment (Nakamura, 1969, Nishimura, 1974, Imoto *et al.*, 2013). Therefore, the relationships between recent lineages most likely reflect paleogeographic relationships between different geographical regions (Ronquist, 1997).

Concerning the recent distribution and endemism of the cyprinoids in the peri-Mediterraenean, several biogeographical scenarios have been proposed. Each of the hypotheses assumed that cyprinoids originated in the Asia and subsequently dispersed into European peninsulas via two routes. The first one occurred during Oligocene and early Miocene, due to the formation of the Ural Mountain, when the freshwater fauna dispersed via river captures and connection in the central Europe and reached the southern European Peninsulas before the formation of Pyrenees (Almaça, 1988, Bănărescu, 1992) (Figure 4-A). Subsequently some species dispersed to the North Africa (Bănărescu, 1989, 1992) via the land bridge in the place of the present strait of Gibraltar. The second hypothesis was proposed by Doadrio (1990) and dispersion supposedly occurred via continental bridge dividing Paratethys and connecting North Africa with Balkanian/Anatolian landmass (Steininger & Rögl, 1984, Perea *et al.*, 2010) (Figure 4-B). According to this hypothesis freshwater fauna dispersed over Northern Africa and

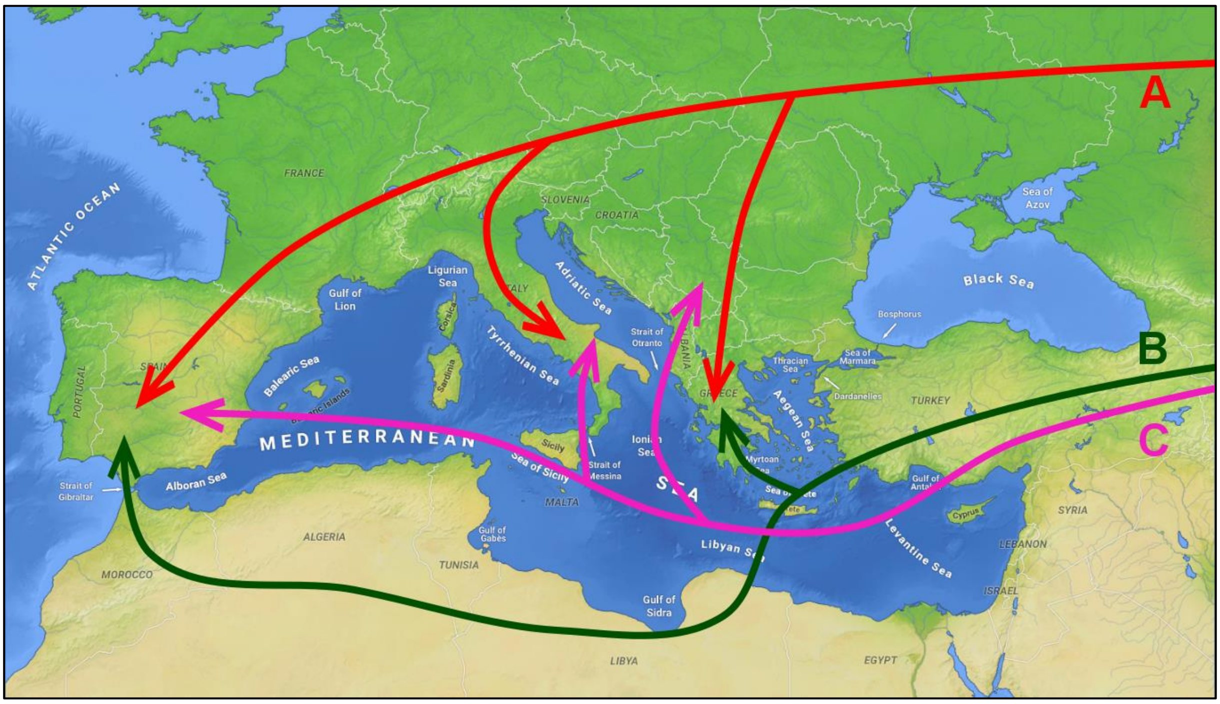

Figure 4. Scheme showing historical dispersion routes proposed for cyprinoids
A = the northern one via Central Europe (Almaça, 1988; Bănărescu, 1992); B = the southern one over North Africa (Doadrio, 1990, Perea *et al.,* 2010); C = via Mediterranean Sea basin during Messinian Salinity Crisis (Bianco, 1990).

colonized the Iberian Peninsula from the south. The second dispersion route is supported also by molecular phylogeny of cyprinids, specifically relatedness of North African cyprinids to Middle-Eastern species (Yang *et al.*, 2015). Bianco (1990) proposed that historical dispersion of cyprinoids is connected with the Lago Mare phase of Messinian Salinity Crisis (5.96 MYA, Krijgsman *et al.* (1992)), during which the Mediterranean Sea nearly dried out following the elevation of strait of Gibraltar (Figure 4-C). Later, the Mediterranean basin was refilled with freshwater and formed water body known as Sarmatian Sea (Hsü *et al.*, 1977). During that time, the freshwater fauna supposedly dispersed via Mediterranean basin into southern European peninsulas and after reopening of strait of Gibraltar (5.33 MYA) alopatrically speciated (Bianco, 1990). However, the last hypothesis was rejected by many authors on the basis of geological and molecular data (Tsigenopoulos *et al.*, 2003, Perea *et al.*, 2010, Yang *et al.*, 2015).

Nevertheless, the historical dispersion and differentiation of cyprinoid fauna is far more complex and is also influenced by the later formed land-bridges connecting geographically isolated regions (such as abovementioned Betic-Kabilian plate, Machordom & Doadrio (2001)). However, all hypotheses concurrently consider the Asian origin of cyprinoids from where different cyprinoid lineages dispersed into peri-Mediterranean.

4 Material and Methods

4.1 Material collection, fixation and identification

Material used in this Ph.D thesis was obtained over years 2014 to 2017. Endemic cyprinids of peri-Mediterranean and several non-endemic species from Czech Republic were collected from 88 localities in nine countries (see Table 2 and Figure 5). A total of 1148 fish specimens of 150 cyprinoid species (Table 3) were examined using standard protocol described by Ergens & Lom (1970) for presence of *Dactylogyrus* parasites. A fin clip was obtained from each processed specimen and preserved in 96% ethanol.

A total of 91 *Dactylogyrus* species were collected from the gills or nasal cavities of 129 endemic and non-endemic cyprinoid species (Table 4). Individual specimens were mounted on slides, covered in mixture of glycerin and ammonium picrate (GAP, Malmberg 1957) and completely flattened under coverslip to exposure taxonomically important characters. For the species determination the sclerotized parts of haptor (i.e. haptoral sclerites) and reproductive organs (male copulatory organ and vaginal armament) were used following Pugachev *et al.* (2009). Identification at the species level was performed using an Olympus BX51 microscope equipped with the phase-contrast optics. *Dactylogyrus* specimens selected for the extraction of DNA and subsequent molecular analyses were bisected using fine needles. The one half (either anterior one with copulatory organs, or posterior one with haptor) was mounted on slide for

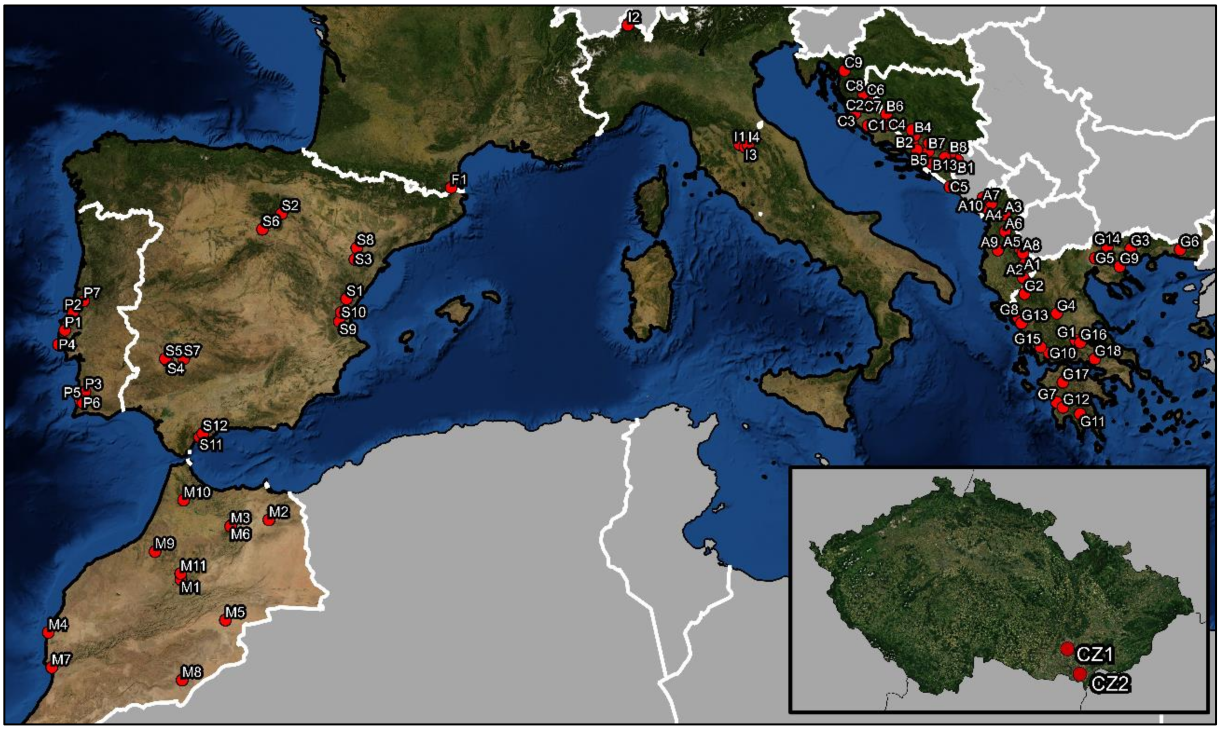

Figure 5. Map of collection localities in the peri-Mediterranean and Czech Republic

morphological identification. The other half was individually preserved in 96% ethanol for extraction of DNA.

Considering delimitation of host specificity for *Dactylogyrus* by Šimková *et al.* (2006b) the modified version taking into account the present classification of cyprinoids (Tan & Armbruster, 2018) was applied in this thesis Table 4. The *Dactylogyrus* species were divided into five classes: (1) the strict specialists parasitizing single cyprinoid species, (2) intermediate specialists parasitizing congeneric host species, (3) transitional generalists parasitizing cyprinoid species belonging to single subfamily, (4) common generalists parasitizing species belonging to single cyprinoid family, and (5) true generalist with not limited hosts range, parasitizing on species from different cyprinoid families.

Sclerotized structures of newly described species were drawn with the aid of drawing attachment and edited in graphic illustration software (Stream Motion v. 1.9.2.). Measurements of sclerotized elements follow Pugachev *et al.* (2009) and marginal hooks were numbered as recommended by Mizelle (1936). Several specimens of each newly described species were after morphometric analysis dehydrated and re-mounted in Canada balsam following Ergens (1969). Type specimens and hologenophores were deposited in the helminthological collection of Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic, in České Budějovice (IPCAS).

To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species were submitted to ZooBank.

Table 2. List of collection localities with coordinates

Country	ID	Locality	Coordinates
Albania	A1	Devoli, Maliq	40°42'57.07"N 20°40'54.06"E
	A2	Osum, Vodice	40°24'13.07"N 20°39'04.04"E
	A3	Fani i Vogel, Reps	41°52'51.01"N 20°04'44.04"E
	A4	Skadar lake, Shiroke	42°03'24.94"N 19°28'07.05"E
	A5	Shkumbini, Perrenjas	41°03'50.09"N 20°33'56.06"E
	A6	Mat, Klos	41°29'37.01"N 20°05'29.04"E
	A7	Kiri	42°08'56.02"N 19°39'42.01"E
	A8	Ohrid lake	40°59'00.66"N 20°38'23.40"E
	A9	Shkumbini, Pajove	41°03'31.07"N 19°51'47.03"E
	A10	Skadar lake, Shegan	42°16'22.09"N 19°23'39.09"E
Bosnia and Herzegovina	B1	Mušnica, Avtovac	43°08'42.05"N 18°35'45.00"E
	B2	Zagorje, Jabuke	43°32'18.53"N 17°12'34.28"E
	B3	Rečina river, near Jelim lake, Hutovo Blato	43°03'39.72"N 17°48'29.30"E
	B4	Šujica, Duvansko Polje	43°42'05.07"N 17°15'50.05"E
	B5	Nezdravica, Trebižat	43°19'00.05"N 17°23'20.01"E
	B6	Bosansko Grahovo, Korana river	44°10'37.00"N 16°23'03.61"E
	B7	Lištica, Polog	43°20'32.09"N 17°41'37.04"E
	B8	Zalomka, Ribari	43°15'26.04"N 18°21'41.05"E
	B9	Krenica lake, Drinovci	43°22'25.00"N 17°19'59.04"E
	B10	Donja Drežnica, Drežnica river	43°31'31.46"N 17°42'51.66"E
	B11	Šujica, Šujičko Polje	43°49'41.43"N 17°10'48.20"E
	B12	Vrijeka, Dabarsko Polje	43°03'32.07"N 18°14'39.04"E
	B13	Zalomka, Nevesinjsko polje	43°12'06.06"N 18°12'21.07"E
Croatia	C1	Bribirske Mostine, Bribišnica	43°55'28.21"N 15°48'45.07"E
	C2	Lovinac, Ričica river	44°22'44.72"N 15°40'15.87"E
	C3	Baštica river, below the Baštica reservoir/Grabovač reservoir	44°11'42.37"N 15°24'32.13"E
	C4	Cetina river, Kosore	43°56'29.78"N 16°26'23.37"E
	C5	Konavočica, Grude	42°31'33.86"N 18°22'04.16"E
	C6	Udbina, Krbava river	44°32'32.00"N 15°46'13.02"E
	C7	Sveti Rok, Obsenica river	44°21'03.64"N 15°40'40.00"E
	C8	Krbavsko polje, Laudonov gaj	44°38'14.33"N 15°40'05.65"E
	C9	Drežnica, Sušik river	45°08'44.13"N 15°04'41.56"E
Czech republic	CZ1	Svratka River	49°05'32.01"N 16°37'11.00"E
	CZ2	Dyje River	48°48'09.04"N 16°50'19.03"E
France	F1	Tech River, le Boulou	42°30'49.80"N, 02°48'40.08"E
Greece	G1	Sperchios, Ypati	38°54'14.33"N 22°17'30.22"E
	G2	Aoos, Kalithea	40°01'16.67"N 20°41'40.19"E
	G3	Angistis, between Alistrati & Drama	41°05'42.08"N 24°00'18.29"E
	G4	Pinios, Rongia - Valamandrio	39°33'07.85"N 21°42'08.02"E
	G5	Gallikos, Mandres, Gallikos basin	40°52'07.33"N 22°53'59.12"E
	G6	Macropotamos river, Filiouri basin	41°04'13.00"N 25°32'52.00"E
	G7	Neda, Gianitsochori	37°23'04.34"N 21°41'24.15"E
	G8	Kokitos, Pagrati	39°26'53.02"N 20°30'03.06"E
	G9	Rihios river, Stavros	40°40'16.34"N 23°39'50.87"E
	G10	Trichonis lake, Panetolio	38°35'20.19"N 21°28'02.68"E
	G11	Evrotas, Sparti	37°05'34.70"N 22°25'34.81"E
	G12	Pamissos, Vasiliko	37°15'17.39"N 21°53'45.15"E
	G13	Acheron, Gliki	39°19'00.05"N 20°36'04.03"E
	G14	flood pools by Struma, Lithopos	41°07'40.41"N 23°16'24.70"E
	G15	Rivio, Amvrakia	38°44'37.68"N 21°11'35.86"E
	G16	channel near Sperchios	38°50'54.60"N 22°25'54.46"E
	G17	Erimantos, Tripotamo	37°52'37.07"N 21°53'15.05"E
	G18	stream in Livadia, Kifisos	38°27'02.12"N 22°53'03.02"E
Italy	I1	canale maestro de la Chiana, Chuisa dei Capannoi, Arno basin	43°29'31.07"N 11°48'39.09"E
	I2	Melezzo River, Masera	46°08'00.45"N, 08°19'20.51"E
	I3	Torrente Cerfone, Intoppo	43°26'12.03"N 11°58'33.00"E

	I4	Torrente Cerfone, Le Ville	43°28'42.00"N 12°04'25.03"E
	I5	Po River, Between Verona & Modena	-
Morocco	M1	Oum Er'Rbia, Chbouka River	32°51'32.09"N 05°37'18.09"W
	M2	Moulouya, Moulouya River	34°24'39.00"N, 02°52'27.03"W
	M3	Sebou, Lahder River (1)	34°15'30.01"N 04°03'52.01"W
	M4	Ksob River	31°27'50.07"N, 09°45'25.03"W
	M5	Zoula Oasis	31°47'31.09"N, 04°14'43.05"W
	M6	Sebou, Lahder River (2)	34°14'32.07"N, 04°03'53.09"W
	M7	Tamrhakht River	30°31'33.06"N 09°38'53.06"W
	M8	Drâa River	30°11'12.02"N, 05°34'47.03"W
	M9	Bouregreg, Grou River	33°35'28.01"N 06°25'43.07"W
	M10	Loukkos River	34°54'57.02"N 05°32'17.02"W
	M11	Oum Er'Rbia, El Borj	33°00'58.07"N 05°37'48.06"W
Portugal	P1	Alcabrichel	39°08'51.33"N 09°14'29.14"W
	P2	Alcoa, Fervenca	39°34'00.94"N 08°59'20.34"W
	P3	Torgal river, Mira basin	37°38'16.76"N 08°37'10.58"W
	P4	Colares	38°47'53.37"N 09°26'14.16"W
	P5	Seixe	37°25'22.41"N 08°44'56.42"W
	P6	tributary of Seixe	37°21'47.87"N 08°40'07.45"W
	P7	Arunca, Mondego basin (Vermoil)	39°51'04.61"N 08°39'19.22"W
Spain	S1	Chico River, flow of Palancia	39°54'09.78"N 00°27'19.66"W
	S2	Tera River	41°54'47.49"N 02°28'44.13"W
	S3	Beceite, Uldemo River	40°50'25.59"N 00°11'38.12"E
	S4	Valencia de las Torres, Retin River	38°24'05.60"N 06°02'40.30"W
	S5	Retin River, near Llera	38°27'10.02"N 06°06'24.99"W
	S6	Ucero River	41°32'49.11"N 03°04'32.50"W
	S7	Peraleda de Zaucejo, Zujar River	38°27'12.02"N 05°31'59.67"W
	S8	upstream Maella, Materraña River	41°06'41.00"N 00°08'05.00"E
	S9	Magro River	39°21'18.85"N 00°40'38.85"W
	S10	Turia River	39°34'46.46"N 00°37'09.63"W
	S11	Benehavis, Guadalmina River	36°31'03.45"N 05°02'25.07"W
	S12	Istán, Verde River	36°36'04.25"N 04°56'15.02"W

ID = code used in the map (Figure 5) and following tables.

Table 3. List of all investigated cyprinoid species included in the study

Cyprinoid species	Loc	N	NP	Accession number
Abramis brama (Linnaeus, 1758)	CZ1	5	3	-
Achondrostoma arcasii (Steindachner, 1866)	S1	15	1	-
	S2	10	1	-
Achondrostoma occidentale (Robalo, Almada, Sousa, Santos, Moreira & Doadrio, 2005)	P1	13	2	-
Achondrostoma oligolepis (Robalo, Doadrio, Almada & Kottelat, 2005)	P2	8	0	-
Alburnoides devolli Bogutskaya, Zupančić & Naseka, 2010	A1	6	1	MK482020
Alburnoides economui Barbieri, Vukić, Šanda & Zogaris, 2017	G1	11	0	KM874634
Alburnoides fangfangae Bogutskaya, Zupančić & Naseka, 2010	A2	7	1	KM874574
Alburnoides ohridanus (Karaman, 1928)	A3	10	1	KM874593
Alburnoides prespensis (Karaman, 1924)	G2	5	1	MF152964
Alburnoides strymonicus Chichkoff, 1940	G3	5	2	KM874618
Alburnoides thessalicus Stephanidis, 1950	G4	12	3	KM874622
*Alburnus arborell*a (Bonaparte, 1841)	I1	10	2	MK482021
Alburnus neretvae Buj, Šanda & Perea, 2010	B1	7	2	GU479867
	B2	10	2	-
Alburnus scoranza Bonaparte, 1845	A11	5	2	MK482022
Aulopyge huegelii Heckel, 1842	B5	14	2	AF287416
Barbus balcanicus Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002	G5	5	3	GQ302793
Barbus barbus (Linnaeus, 1758)	CZ1	12	3	AY331021
Barbus caninus Bonaparte, 1839	I2	10	0	MN961173
Barbus cyclolepis Heckel, 1837	G6	3	2	AF090782
Barbus haasi Mertens, 1925	S3	4	1	AF334101
Barbus meridionalis Risso, 1827	F1	11	1	MN961174
Barbus peloponnesius Valenciennes, 1842	G7	8	1	MK482024
	G8	5	3	MK482023
Barbus plebejus Bonaparte, 1839	C1	7	3	MK482025
Barbus prespensis Karaman, 1924	A5	5	1	GQ302762
	G2	5	4	GQ302763
Barbus rebeli Koller, 1926	A6	7	3	GQ302779
Barbus sp.	A7	6	1	GQ302774
Barbus sperchiensis Stephanidis, 1950	G1	4	1	AF090783
Barbus strumicae Karaman, 1955	G9	5	1	AF090784
Barbus tyberinus Bonaparte, 1839	I3	5	1	AF397300
Carassius gibelio (Bloch, 1782)	CZ2	5	1	-
	C3	10	2	-
Carasobarbus fritschii (Günther, 1874)	M1	14	3	MN961175
	M2	1	1	MN961177
	M3	7	2	MN961176
Chondrostoma knerii Heckel, 1843	B3	5	2	MG806656
Chondrostoma nasus (Linnaeus, 1758)	CZ1	5	1	-
Chondrostoma ohridana Karaman, 1924	G2	4	3	MK482026
Chondrostoma phoxinus Heckel, 1843	B4	11	1	MK482027
Chondrostoma vardarense Karaman, 1928	G3	5	2	MK482028
	G4	1	2	-
Cyprinus carpio Linnaeus, 1758	CZ1	3	1	-
Delminichthys adspersus (Heckel, 1843)	B5	10	1	HM560089
Iberochondrostoma almacai (Coelho, Mesquita & Collares-Pereira, 2005)	P3	19	1	-
Iberochondrostoma lemingii (Steindachner, 1866)	S4	15	0	-
Iberochondrostoma lusitanicum (Collares-Pereira, 1980)	P4	15	0	-
Iberocypris alburnoides (Steindachner, 1866)	S5	12	1	-
Luciobarbus albanicus (Steindachner, 1870)	G10	9	1	AY004723
Luciobarbus bocagei (Steindachner, 1864)	P4	6	2	MN961178
	S6	10	1	-
Luciobarbus comizo (Steindachner, 1864)	S7	5	3	KY457956
Luciobarbus graecus (Steindachner, 1895)	G1	10	1	AF090786
Luciobarbus graellsii (Steindachner, 1866)	S3	1	2	MN961180
	S8	5	4	MN961179
Luciobarbus guercifensis Doadrio, Perea & Yahyahoui, 2016	M2	8	0	KU257526
Luciobarbus guiraonis (Steindachner, 1866)	S9	6	3	MN961181

	S10	4	2	MN961182
Luciobarbus ksibi (Boulenger, 1905)	M1	9	2	MN961183
	M4	6	2	MN961184
Luciobarbus lepineyi (Pellegrin, 1939)	M5	8	3	MN961185
Luciobarbus maghrebensis Doadrio, Perea & Yahyaoui, 2015	M6	10	2	MN961186
Luciobarbus massaensis (Pellegrin, 1922)	M7	11	1	MN961187
Luciobarbus microcephalus (Almaça, 1967)	S7	5	0	KY457954
Luciobarbus pallaryi (Pellegrin, 1919)	M8	7	1	AY004745
Luciobarbus rabatensis Doadrio, Perea & Yahyaoui, 2015	M9	9	1	MN961188
Luciobarbus rifensis Doadrio, Casal-Lopez & Yahyaoui, 2015	M10	10	1	MN961189
Luciobarbus sclateri (Günther, 1868)	P3	5	2	KY457853
	S11	10	2	KY457882
Luciobarbus yahyaouii Doadrio, Casal-Lopez & Perea, 2016	M2	11	1	MN961190
Luciobarbus zayanensis Doadrio, Casal-López & Yahyaoui, 2016	M11	3	2	MN961191
Pachychilon macedonicum (Steindachner, 1892)	G4	8	1	MG806671
Pachychilon pictum (Heckel & Kner, 1858)	A8	4	2	MK482029
	G2	5	5	-
Parachondrostoma arrigonis (Steindachner, 1866)	S9	3	0	-
Parachondrostoma miegii (Steindachner, 1866)	S3	12	1	-
Parachondrostoma turiense (Elvira, 1987)	S10	18	1	-
Pelasgus laconicus (Kottelat & Barbieri, 2004)	G11	13	1	MG806673
Pelasgus marathonicus (Vinciguerra, 1921)	G1	11	0	MG806674
Pelasgus stymphalicus (Valenciennes, 1844)	G12	5	0	HM560109
Pelasgus thesproticus (Stephanidis, 1939)	G7	5	0	-
	G13	1	0	MK482030
Phoxinellus alepidotus Heckel, 1843	B6	12	1	MG806680
Phoxinellus pseudalepidotus Bogutskaya & Zupančić, 2003	B7	10	1	MG806681
Phoxinus bigerri Kottelat, 2007	S6	12	1	MK482031
Phoxinus lumaireul Schinz, 1840	C2	11	0	-
Phoxinus sp.	B8	14	1	MK482032
Protochondrostoma genei (Bonaparte, 1839)	I4	9	2	AY568621
Pseudochondrostoma duriense (Coelho, 1985)	S6	9	2	-
Pseudochondrostoma polylepis (Steindachner, 1864)	P2	10	1	-
	P4	15	1	
Pseudochondrostoma wilcomi (Steindachner, 1866)	S12	11	0	-
Pterocapoeta maroccana Günther, 1902	M11	3	1	KF876030
Rhodeus meridionalis Karaman, 1924	G4	15	1	-
Rutilus aula (Bonaparte, 1841)	C3	10	1	FJ824719
Rutilus basak (Heckel, 1843)	B9	13	4	FJ824720
Rutilus lacustris (Pallas, 1814)	G14	3	4	MG806693
Rutilus ohridanus (Karaman, 1924)	A4	4	5	HM156741
Rutilus panosi Bogutskaya & Iliadou, 2006	G15	5	0	MG806694
Rutilus rubilio (Bonaparte, 1837)	I3	10	4	MK482033
Rutilus rutilus (Linnaeus, 1758)	CZ1	5	3	-
Rutilus sp.	G16	4	0	MK482034
Scardinius acarnanicus Economidis, 1991	G10	4	0	MG806697
Scardinius dergle Heckel & Kner, 1858	C1	10	1	MK482035
Scardinius plotizza Heckel & Kner, 1858	B3	7	3	MK482036
Squalius aradensis (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998)	P5	5	1	-
	P6	6	0	-
Squalius carolitertii (Doadrio, 1988)	P7	15	3	-
Squalius cephalus (Linnaeus, 1758)	CZ1	5	2	-
	B6	4	2	-
Squalius illyricus Heckel & Kner, 1858	C4	2	1	MG806702
Squalius keadicus (Stephanidis, 1971)	G11	5	0	KY070419
Squalius laietanus Doadrio, Kottelat, de & Sostoa, 2007	S8	5	0	-
Squalius lucumonis (Bianco, 1983)	I3	10	4	MK482037
Squalius malacitamus Doadrio & Carmona, 2006	S11	10	0	MG806704
Squalius microlepis Heckel, 1843	B7	1	0	-
Squalius orpheus Kottelat & Economidis, 2006	G9	4	1	MK482038
Squalius pamvoticus (Stephanidis, 1939)	G13	6	1	KY070381
Squalius peloponnensis (Valenciennes, 1844)	G12	5	1	KY070368

Squalius platyceps Zupančić, Marič, Naseka & Bogutskaya, 2010	A8	5	2	MK482039
Squalius prespensis (Fowler, 1977)	A9	4	2	MK482041
	G2	6	3	MK482040
Squalius pyrenaicus (Günther, 1868)	P4	5	1	MK482042
	S7	5	1	-
Squalius sp.	G10	2	2	-
Squalius squalus (Bonaparte, 1837)	B10	10	4	MG806710
	I5	11	3	MK482043
Squalius svallize Heckel & Kner, 1858	C5	15	1	MG806711
Squalius tenellus Heckel, 1843	B3	2	2	MG806712
	B11	11	2	-
Squalius torgalensis (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998)	P3	10	1	-
Squalius valentinus Doadrio & Carmona, 2006	S9	10	0	-
Squalius vardarensis Karaman, 1928	G1	1	0	MK482045
	G5	4	3	MK482044
Squalius zrmanjae Karaman, 1928	C6	10	0	MK482046
Telestes alfiensis (Stephanidis, 1971)	G17	5	1	AF090765
Telestes beoticus (Stephanidis, 1939)	G18	8	0	MK482047
Telestes croaticus (Steindachner, 1866)	C7	12	0	MG806715
Telestes dabar Bogutskaya, Zupančić, Bogut & Naseka, 2012	B12	3	1	MG806716
Telestes fontinalis (Karaman, 1972)	C8	13	2	HM560215
Telestes karsticus Marčić & Markovčić, 2011	C9	10	2	JN188372
Telestes metohiensis (Steindachner, 1901)	B13	5	2	MK482048
Telestes montenigrinus (Vukovic, 1963)	A10	10	3	MG806718
Telestes muticellus (Bonaparte, 1837)	I3	7	2	MK482049
Telestes pleurobipunctatus (Stephanidis, 1939)	G8	6	1	MK482050
Tropidophoxinellus helenicus (Stephanidis, 1971)	G15	9	0	HM560232
Tropidophoxinellus spartiaticus (Schmidt-Ries, 1943)	G7	5	1	AF090777
Vimba vimba (Linnaeus, 1758)	CZ1	5	3	-

Loc = codes of localities corresponding to Table 2 and Figure 5; N = number of parasitologically processed specimens; NP = number of collected *Dactylogyrus* species from given host; Accession number = representative cytochrome *b* gene sequence in GenBank.

Table 4. List of all collected *Dactylogyrus* species with the respective hosts.

Dactylogyrus species Authority	HS	Hosts in the presented studies	18S rDNA	28S rDNA
D. alatus Linstow, 1878	3	*A. neretvae*	MG792842	MG792956
D. anchoratus (Dujardin, 1845)	3	*C. gibelio*	KY859795	KY863555
D. andalousiensis El Gharbi, Renaud & Lambert, 1993	2	*L. comizo, L. sclateri*	MN365672	MN338207
D. atlasensis El Gharbi, Birgi & Lambert, 1994	1	*L. lepineyi*	KY629337	KY629356
D. auriculatus (Nordmann, 1832)	2	*A. brama*	MG792838	MG792952
D. balistae Simon-Vicente, 1981	2	*L. bocagei*	KY629344	MN338205
D. balkanicus Dupont & Lambert, 1986	2	*B. plebejus, B. prespensis, B. rebeli*	KY201093	KY201107
D. benhoussai Rahmouni, Řehulková & Šimková, 2017	1	*L. yahyaouii*	MN974254	MN973815
D. bicornis Malewitzkaja, 1941	2	*R. meridionalis*	-	KY629345
D. bocageii Alvarez Pellitero, Simon Vicente & Gonzalez Lanza, 1981	3	*L. bocagei, L. comizo, L. graellsii, L. sclateri*	MN365671	KY629347
D. borealis Nybelin, 1937	2	*Phoxinus* sp., *P. bigerri*	KY629343	KY629372
D. borjensis El Gharbi, Birgi & Lambert, 1994	1	*L. zayanensis*	MN974257	MN973819
D. caballeroi Prost, 1960	2	*R. ohridanus, R. rutilus*	MG792902	MG793018
D. carpathicus Zachvatkin, 1951	2	*B. barbus*	KY201098	KY201111
D. caucasicus Mikailov & Shaova, 1973	2	*A. devolli, A. fangfangae, A. prespensis*	MG792847	MG792961
D. cornu Linstow, 1878	3	*V. vimba*	KY629342	KY629371
D. crivellius Dupont & Lambert, 1986	2	*B. balcanicus, B. peloponnesius, B. plebejus, B. prespensis, B. rebeli, Barbus* sp., *B. tyberinus*	KY201094	KY201108
D. crucifer Wagener, 1857	2	*R. lacustris, R. rutilus*	MG792898	MG793014
D. difformis Wagener, 1857	2	*S. plotizza*	MG792908	MG793025
D. difformoides Glaeser & Gussev, 1967	2	*S. plotizza*	MG792909	MG793026
D. dirigerus Gussev, 1966	2	*C. ohridana, C. vardarense*	MG792876	MG792991
D. dyki Ergens & Lucky, 1959	2	*B. balcanicus, B. barbus, B. cyclolepis, D. peloponnesius, B. prespensis, B. rebeli, B. sperchiensis, B. strumicae*	KY201095	KY201109
D. ergensi Molnar, 1964	3	*C. knerii, C. ohridana, D. vardarense, P. genei, S. lucumonis, S. squalus*	MG792874	MG792989
D. erhardovae Ergens, 1970	2	*R. aula, R. basak, R. ohridanus*	MG792893	MG793009
D. extensus Müller & Van Cleave, 1932	1	*C. carpio*	KM277459	AY553629
D. fallax Wagener, 1857	3	*C. nasus, R. rutilus, V. vimba*	MG792906	MG793023
D. falsiphallus Rahmouni, Řehulková & Šimková, 2017	1	*L. maghrebensis*	MN974253	MN973813
D. fimbriphallus El Gharbi, Birgi & Lambert, 1994	2	*L. lepineyi, L. massaensis, L. pallaryi*	KY629332	KY629357
D. folkmanovae Ergens,1956	2	*S. cephalus, Squalius* sp., *S. orpheus, S. platyceps, S. prespensis, S. squalus, S. vardarensis*	MG792921	MG793040
D. formosus Kulwiec, 1927	2	*C. gibelio*	MG792869	MG792984
D. ivanovichi Ergens, 1970	1	*P. pictum*	MG792883	MG792999
D. izjumovae Gussev, 1966	2	*S. dergle, S. plotizza*	MG792910	MG793027
D. ksibii El Gharbi, Birgi & Lambert, 1994	2	*L. ksibi, L. rabatensis*	MN974251	MN973811
D. kulundrii El Gharbi, Birgi & Lambert, 1994	2	*C. fritschii*	KY629336	KY629354
D. legionensis Gonzalez Lanza & Alvarez Pellitero, 1982	2	*L. graellsii, L. guiraonis*	MN365678	MN338210

D. lenkoranoïdes El Gharbi, Renaud & Lambert, 1993	3	*B. haasi, L. graellsii*	MN365676	MN338211
D. linstowoïdes El Gharbi, Renaud & Lambert, 1993	2	*L. guiraonis*	KY629329	KY629349
D. malleus Linstow, 1877	2	*B. barbus*	KY201099	KY201112
D. maroccanus El Gharbi, Birgi & Lambert, 1994	4	*C. fritschii, L. ksibi, L. zayanensis, P. maroccana*	KY629333	KY629355
D. martinovici Ergens, 1970	1	*P. pictum*	MG792884	MG793000
D. mascomai El Gharbi, Renaud & Lambert, 1993	3	*L. bocagei, L. graellsii, L. guiraonis*	MN365680	MN338215
D. minor Wagener, 1857	2	*A. scoranza*	MG792848	MG792962
D. nanoides Gussev, 1966	2	*S. cephalus, S. prespensis, S. squalus*	MG792923	MG793045
D. nanus Dogiel & Bychowsky, 1934	3	*R. rubilio*	MK434933	MK434953
D. omenti Benovics, Kičinjaová & Šimková, 2017	1	*A. huegelii*	KY201091	KY201105
D. parvus Wegener, 1910	2	*A. scoranza*	MG792849	MG792963
D. petenyi Kastak, 1957	2	*B. balcanicus, B. cyclolepis, B. peloponnesius*	KY201097	KY201113
D. petkovici Ergens, 1970	1	*P. pictum*	MG792886	MG793002
D. polylepidis Alvarez Pellitero, Simon Vicente & Gonzalez Lanza, 1981	3	*A. arcasii, P. duriense, S. carolitertii*	MN365664	MN338198
D. prespensis Karaman, 1924	2	*B. prespensis*	KY201096	KY201110
D. prostae Molnar, 1964	2	*S. cephalus, Squalius* sp., *S. lucumonis, S. pamvoticus, S. prespensis, S. squalus*	MG792924	MG793042
D. rarissimus Gussev, 1966	3	*A. arborella, A. neretvae, P. laconicus, R. basak, R. lacustris, R. ohridanus, R. rubilio, T. alfiensis, T. dabar, D. fontinalis, T. metohiensis*	MG792899	MG793015
D. rosickyi Ergens, 1970	1	*P. pictum*	MG792888	MG793004
D. rutili Glaeser, 1965	2	*R. basak, R. lacustris, R. ohridanus*	MG792900	MG793016
D. rysavyi Ergens, 1970	2	*A. thessalicus*	MG792851	MG792965
D. scorpius Rahmouni, Řehulková & Šimková, 2017	1	*L. rifensis*	MN974256	MN973818
D. sekulovici Ergens, 1970	1	*P. pictum*	MG792889	MG793005
D. soufii Lambert, 1977	2	*T. montenigrinus*	MG792946	MG793061
D. sphyrna Linstow, 1878	3	*R. basak, R. ohridanus, R. rubilio*	MG792905	MG793021
D. suecicus Nybelin, 1937	2	*R. lacustris, T. montenigrinus*	MG792901	MG793017
D. tissensis Zachvatkin, 1951	2	*A. thessalicus*	MG792852	MG792966
D. varius Rahmouni, Řehulková & Šimková, 2017	1	*L. maghrebensis*	MN974255	MN973814
D. vastator Nybelin, 1924	4	*A. huegelii, B. plebejus, C. gibelio*	KY201092	KY201106
D. vistulae Prost, 1957	5	*A. ohridanus, A. strymonicus, A. thessalicus, C. ohridana, C. phoxinus, C. vardarense, P. alepidotus, P. pseudalepidotus, P. genei, R. rubilio, S. illyricus, S. lucumonis, S. peloponnensis, S. platyceps, S. prespensis, S. squalus, S. svallize, S. tenellus, S. vardarensis, T. fontinalis, T. karsticus, T. metohiensis, T. montenigrinus, T. muticellus, T. pleurobipunctatus*	KY629340	KY629369
D. volutus El Gharbi, Birgi & Lambert, 1994	1	*C. fritschii*	KY629334	KY629353
D. vranoviensis Ergens, 1956	2	*S. squalus, S. vardarensis*	MG792931	MG793048
D. wunderi Bychowsky, 1931	1	*A. brama*	KY629375	AJ564164
D. yinwenyingae Gussev, 1962	4	*S. lucumonis*	MK434939	MK434959
D. zandti Bychowsky, 1933	1	*A. brama*	MG792839	MG792953
D. zatensis El Gharbi, Birgi & Lambert, 1994	1	*C. fritschii*	KY629335	KY629352
Dactylogyrus sp. 1	1	*S. tenellus*	MG792933	MG793050

Dactylogyrus sp. 2	1	*L. graecus*	KY201101	KY201115
Dactylogyrus sp. 3	1	*L. alabnicus*	KY201100	KY201114
Dactylogyrus sp. 4	1	*D. adspersus*	MG792881	MG792995
Dactylogyrus sp. 5	1	*P. macedonicum*	MG792882	MG792998
Dactylogyrus sp. 6	1	*T. spartiaticus*	MG792950	MG793065
Dactylogyrus sp. 7	1	*C. knerii*	MG792871	MG792986
Dactylogyrus sp. 8	1	*T. karsticus*	MG792942	MG793057
Dactylogyrus sp. 9	1	*T. montenigrinus*	MG792947	MG793062
Dactylogyrus sp. 10	1	*T. muticellus*	MK434944	MK434964
Dactylogyrus sp. 11 -	2	*S. aradensis. S. torgalensis*	MN365691	MN338225
Dactylogyrus sp. 12 -	1	*A. occidentale*	MN365666	MN338200
Dactylogyrus sp. 13 -	1	*I. almacai*	MN365669	MN338203
Dactylogyrus sp. 14 -	1	*S. torgalensis*	MN365697	MN338231
Dactylogyrus sp. 15 -	1	*I. alburnoides*	MN365670	MN338204
Dactylogyrus sp. 16 -	1	*P. polylepis*	MN365690	MN338224
Dactylogyrus sp. 17 -	2	*S. carolitertii, S. pyrenaicus*	MN365694	MN338228
Dactylogyrus sp. 18 -	2	*P. miegii, P. turiense*	MN365686	MN338220
Dactylogyrus sp. 19 -	1	*P. duriense*	MN365689	MN338223
Dactylogyrus sp. 20 -	1	*A. occidentale*	MN365667	MN338201
Dactylogyrus sp. 21 -	1	*S. carolitertii*	MN365693	MN338227

HS = level of host specificity: 1 = the strict specialist, 2 = intermediate specialist, 3 = transitional generalist, 4 = common generalist, 5 = true generalist; 18S rDNA, 28S rDNA = accession numbers to representative sequences in GenBank

4.2 DNA extraction, amplification and sequencing of parasites

Bisected *Dactylogyrus* preserved in the ethanol were dried using vacuum centrifuge. Extraction of whole genomic DNA was performed using DNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following protocol provided by manufacturer. Up to four genetic markers were used for *Dactylogyrus*. The partial gene coding 18S rRNA, the entire ITS1, and partial gene coding 5.8S rRNA (hereinafter abbreviated as 18S, ITS1 and 5.8S) were amplified using the primers S1 (forward, 5′- ATTCCGATAACGAACGAGACT- 3′) and IR8 (reverse, 5′- GCTAGCTGCGTTCTTCATCGA- 3′), which anneal to the segments of DNA coding 18S and 5.8S rRNA, respectively (Šimková *et al.*, 2003). Alternatively, for the amplification of the same region, combination of primers S1 and Lig5.8R (5'-GATACTCGAGCCGAGTGATCC -3') was used, last primer anneals also to the 5.8S region (Šimková *et al.* 2003, Blasco-Costa *et al.* 2012). Amplification reactions followed protocols optimized in Papers III and VII, respectively. For the part of the gene coding 28S rRNA (hereinafter abbreviated as 28S), DNA was amplified using the forward primer C1 (5'-ACCCGCTGAATTTAAGCA-3') and reverse primer D2 (5'-TGGTCCGTGTTTCAAGAC-3') (Hassouna *et al.* 1984), following the PCR protocol optimized by Šimková *et al.* (2006a). The PCR products (~1,000 for 18S, ITS1, and 5.8S, and ~800 bp for partial 28S) were checked on 1% agarose gel and purified using the ExoSAP- IT kit (Ecoli, Bratislava, Slovakia) following the standard protocol. The purified products were directly sequenced using the same primers as for PCR and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Prague, Czech Republic). Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Prague, Czech Republic).

For fish hosts, DNA extraction and all laboratory procedures were carried out by collaborating laboratory in the Charles University in Prague. The complete mtDNA cytochrome *b* gene of length 1140 bp was amplified using primers GluF (forward, 5'-AACCACCGTTGTATTCAACTACAA-3') and ThrR (reverse, 5'-ACCTCCGATCTTCGGATTACAAGACCG-3') according to Machordom and Doadrio (2001). Amplification reaction followed protocol optimized by Šanda *et al.* (2008). Sequencing was carried out by the Macrogene Service Centre (Seoul, South Korea) using the amplification primers.

4.3 Phylogenetic and cophylogenetic analyses

DNA sequence alignments were built of concatenating either all four genetic markers, partial genes coding 18S and 28S rDNA, or using single genetic marker. Homologue sequences were aligned using Fast Fourier transform algorithm in MAFFT (Katoh *et al.*, 2002), or alternatively using ClustalW algorithm (Thompson *et al.*, 194). Gaps, and hypervariable, ambiguously aligned, regions were removed from final alignments using Gblocks v. 0.91 (Talavera & Castresana, 2007). The data were treated as partitioned and appropriate evolutionary model was selected for each gene segment using jModelTest v 2.1.10 (Guindon & Gascuel, 2003, Darriba *et al.*, 2012). Phylogenetic analyses using maximum likelihood were computed employing either RaxML v 8.1.12 (Stamatakis 2006, 2014), or PhyML v 3.0 (Guindon *et al.*, 2010). Phylogenetic analyses of Bayesian inference were carried out in MrBayes v 3.2 (Ronquist *et al.*, 2012). Phylogenetic analyses based on minimum evolution algorithm were performed using PAUP 4b10 (Swofford, 2002). Mapping of specific characters (e.g. morphological features, or host distribution) into phylogenetic trees was performed in Mesquite v 3.2 (Maddison & Maddison, 2019).

Tanglegrams connecting host and parasite phylogenetic trees via host-parasite associations were built with TreeMap v 3.0b (Charleston, 2012). ParaFit implemented in CopyCat (Meier-Kolthoff *et al.*, 2007) was employed for distance-based cophylogenetic analyses (Legendre *et al.*, 2002). This method using the patristic distances calculated for parasite and hosts phylogenies allows us to assess the significance of global fit and individual coevolutionary links. The event-based cophylogenetic analyses were performed in Jane 4.0 (Conow *et al.*, 2010). Eleven different cost schemes were tested to assess the importance of each coevolutionary event in host-parasite system investigated.

5 Results and Discussion

The Results and Discussion chapters are presented as the compilation of papers (published or accepted for publication) and manuscripts submitted to scientific journals. When submitting Ph.D thesis, a total of four papers were published, one was accepted for publication and two manuscripts were under peer-review process in scientific journals. Despite that papers and manuscripts are numbered chronologically, this chapter is thematically divided into three sections; each compiling results of several papers and/or manuscripts. The first section comprises two papers and one manuscript, and is focused on the diversity, distribution, phylogeny, and phylogeography of endemic and non-endemic *Dactylogyrus* species in the Balkan Peninsula. The second section comprises two papers and focusses on diversity, endemism, phylogeny and phylogeography of *Dactylogyrus* species parasitizing endemic cyprinoids in eastern peri-Mediterranean (Iberian Peninsula). The last section comprises one paper and one manuscript investigating cophylogenetic relationships between endemic peri-Mediterranean cyprinoids and their *Dactylogyrus* parasites, and tackles different phylogeographic scenarios in this host-parasite system, with special focus on application of parasites as tool for studying historical dispersion of their hosts. Full text of all papers are included as the appendices.

Section I – Phylogeny of *Dactylogyrus* in the Balkans

Paper I

Benovics M., Kičinjaová M. L. & Šimková A. (2017) The phylogenetic position of the enigmatic Balkan *Aulopyge huegelii* (Teleostei: Cyprinidae) from the perspective of host-specific *Dactylogyrus* parasites (Monogenea) with a description of *Dactylogyrus omenti* n. sp. *Parasites & Vectors* 10: 547. doi: 10.1186/s13071-017-2491-z

Paper III

Benovics M., Desdevises Y., Vukić J., Šanda R. & Šimková, A. (2018) The phylogenetic relationships and species richness of host-specific *Dactylogyrus* parasites shaped by the biogeography of Balkan cyprinids. *Scientific Reports* 8: 13006. doi: 10.1038/s41598-018-31382-w

Paper VI

Řehulková E., **Benovics M.** & Šimková A. (submitted) Seven new species of *Dactylogyrus* Diesing, 1850 (Platyhelminthes: Monogenea) from the gills of endemic cypriniform fishes in the Balkan Peninsula: an integrated morphological and molecular approach to species delimitation. *Parasitology Research* (November 2019).

Balkan Peninsula is generally considered as a hotspot of biodiversity. Due to formation of the landmass and historical rearrangement of Dessaretes lake system this Peninsula harbours remarkably high number of endemic species (Sušnik *et al.*, 2007, Abell *et al.*, 2008, Schultheiss *et al.*, 2008, Wagner & Wilke, 2011, Oikonomou *et al.*, 2014). Moreover, the highest number of highly diversified cyprinoid genera is present in the Balkans where majority of endemic species have incredibly small distribution range, often limited to only single river or lake system (Kottelat & Freyhof, 2007). However, species diversity and degree of endemism of cyprinoids is the most likely linked not only to formation of region, but also sequential colonization of the Peninsula by cyprinoids over multiple dispersion events (i.e. colonization waves). Such gradual colonization of the Balkan region potentially introduced also evolutionary divergent lineages of parasites.

Since the parasitological data on the endemic cyprinoid fauna are scarce and comprises of only small number of outdated papers (e.g. Dupont & Lambert, 1986, Stojanovski *et al.*, 2005, 2012) we focused on these fish and investigated degree of endemism, distribution, and diversity of their host specific monogeneans. Using molecular phylogenetic approach we assessed the relationships between endemic *Dactylogyrus* to congeners parasitizing cyprinoids with wide distribution range in the Europe (e.g. *Rutilus rutilus* or *Squalius cephalus*).

Overall composition of *Dactylogyrus* communities parasitizing endemic cyprinoids appears to be species poorer in comparison to communities in the central Europe (Paper III). While fish hosts with wide distribution range in Europe may harbour up to 10 *Dactylogyrus* species (e.g. Šimková *et al.*, 2000, Seifertová *et al.*, 2008), we found only up to five *Dactylogyrus* species from single host species in the Balkans. In general, endemic cyprinoids with limited distribution range were parasitized by host-specific endemic *Dactylogyrus*. Furthermore, the high intraspecific genetic diversity was observed in the Balkan *Dactylogyrus* species. Subsequent species delimitation analyses suggested surprisingly higher species diversity on the molecular level in contrast to traditional morphological approach. Therefore, we assume that *Dactylogyrus* species with wide host range actually represent the complexes of cryptic species. Phylogenetic reconstruction divided *Dactylogyrus* species into four major clades, from which one encompassed majority of investigated species form the Balkans and central Europe. Interestingly, some endemic cyprinoids (e.g. *Pachychilon pictum*) harboured phylogenetically divergent host-specific *Dactylogyrus* species, each of them representative of different lineage. Extrapolating from such observation we hypothesized, that several endemic *Dactylogyrus* species originated from host switching of phylogenetically and geographically distant host species during their secondary contact of cyprinoid species via underground river connections.

Subsequently we selected three generalist *Dactylogyrus* species which exhibit different level host specificity (*D. rarissimus* parasitizing on phylogenetically related non-congeners, *D. folkmanovae* parasitizing only on congeneric hosts (i.e. *Squalius* spp.), and *D. vistulae* – true generalist species parasitizing on wide range of phylogenetically non-related cyprinoid genera) to investigate whether the interpopulation genetic distances correlate with the geographic distances between populations (Paper III). In general, we found the correlation; however, the minor discrepancies were observed in the structure of *D. rarissimus* populations, suggesting influence of introduction of non-native fish hosts into new region, possibly promoting host switching of parasites.

Special focus was given to cyprinid species *Aulopyge huegelii* (Paper I) which is one of the remarkable Balkan endemics with the unresolved phylogenetic position to other cyprinids (e.g. Tsigenopoulos *et al.*, 2003, Wang *et al.*, 2013, Yang *et al.*, 2015). Although this species was previously quite abundant, in recent years, populations of *A. huegelii* have been declining and nowadays this species is *A. huegelii* listed among endangered species in the Balkans

(Mrakovčić *et al.*, 2006). *Aulopyge huegelii* putatively represents a descendant of the first colonization wave of cyprinoids into the Balkans, and Mediterranean region in general (Tsigenopoulos & Berrebi 2000). On the basis of morphological characters and also supported by molecular data, two *Dactylogyrus* species were identified in *A. huegelii*. The first one, *D. vastator* is species commonly identified in *Carassius* spp. and *Cyprinus carpio*. Using molecular data, this *Dactylogyrus* species was compared to *D. vastator* specimens from different regions and hosts in Eurasia. Genetic distances revealed that population of *D. vastator* from *A. huegelii* is the genetically identical with population of *D. vastator* of Balkan *C. gibelio* and genetically more similar to *D. vastator* population from *C. carpio* in central Europe, rather than *D. vastator* population from central European *C. gibelio*. Thus, we hypothesized that *D. vastator* only recently host switched to *A. huegelii* from non-congeneric cyprinids in the Europe. The second species was newly described in this study as *D. omenti* which is according to our subsequent investigation host-specific for *A. huegelii*. The phylogenetic reconstruction based on three molecular markers placed *D. omenti* within *Dactylogyrus* species exhibiting host specificity to *Barbus* species. While the phylogenetic position of *D. omenti* was not fully resolved, on the basis of morphology *D. omenti* resembles *Dactylogyrus* species parasitizing Middle-Eastern cyprinids, suggesting historical contact between species currently living in allopatry and common ancestor of *A. huegelii* and Middle-Eastern cyprinids.

In addition to *D. omenti* we described following seven new *Dactylogyrus* species from following endemic Balkan cyprinoids: *Luciobarbus albanicus*, *L. graecus*, *Pachychilon macedonicum*, *Telestes karsticus*, *Tropidophoxinellus spartiaticus*, *Delminichthys adspersus*, and *Chondrostoma knerii* (Paper VI). Each newly described species was supported by species delimitation analyses within the previous study (Paper III). The most interesting finding is the evidence of two pseudocryptic species from *Luciobarbus* spp. which were described from the only two representatives of genus *Luciobarbus* in the Balkans. Two *Dactylogyrus* species were on the morphological basis nearly indistinguishable, and both were morphologically similar to *Dactylogyrus* species host-specific to North-west African and Middle-Eastern cyprinids (i.e. all these *Dactylogyrus* species share haptoral elements of same morphological type). However, both new species significantly differed on the molecular level, and, in contrast to their cyprinid hosts, were phylogenetically closely associated (sister species), suggesting common evolutionary origin of two *Dactylogyrus* species parasitizing *Luciobarbus* in the Balkans.

Whereas the Balkan Peninsula represents region with high research interest, endemic *Dactylogyrus* fauna appears to be still underexplored. The reason behind limited knowledge about *Dactylogyrus* diversity (and overall monogeneans) may be that the species were previously described solely on the basis of morphological characters without inclusion of any molecular data. Moreover, the *Dactylogyrus* parasites (and many other monogeneans) represent a group where common morphological approach for species delimitation has not full informative value for species delimitation even for the most experienced taxonomists. While the haptor morphology is generally considered as the most important character for resolving phylogenetic relationships in *Dactylogyrus* species, copulatory organs represent rapidly evolving and highly diverse characters commonly used for species determination in *Dactylogyrus*. Concluding from our studies not only morphological characters are important for species delineation, it is also important to take into consideration molecular data of species. The addition of molecular approach may reveal complexes of initially unrecognized species in which posteriori detailed morphological examination may support their existence (such as two species from *L. albanicus* and *L. graecus*).

Furthermore, our results suggest that *Dactylogyrus* parasites may provide important information about historical secondary contacts of fishes which seemingly live in allopatry for long time. Moreover, our studies highlight importance of conservation management and potential threat of endemic fish fauna resulting from introduction of non-native species to new regions as non-native parasite species are often introduced with their hosts.

Section II - Phylogeny of *Dactylogyrus* in the eastern peri-Mediterranean

Paper II

Šimková A., **Benovics M.**, Rahmouni I. & Vukić J. (2017) Host-specific *Dactylogyrus* parasites revealing new insights on the historical biogeography of Northwest African and Iberian cyprinid fish. *Parasites & Vectors* 10: 589. doi: 10.1186/s13071-017-2521-x

Paper V

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* (In press). doi: 10.1017/S0031182020000050

The eastern peri-Mediterranean (i.e. Iberian Peninsula and North-West Africa) is also inhabited by high number of endemic cyprinid species (especially *Luciobarbus* spp.). The important role in the colonization of Iberian Peninsula and radiation of endemic species herein played elevation of tectonic plates connecting southern Iberia with the North-West Africa during Miocene. *Luciobarbus* of Iberian Peninsula and *Luciobarbus* of the North-West Africa formed two phylogenetic lineages (Yang *et al.*, 2015). Nevertheless, different dispersion routes were proposed explaining current distribution of recent cyprinoid lineages living in this area. From these two regions, exceptionally high degree of endemism is in Iberian Peninsula, which is the most likely result of its historical formation and geographic isolation of the landmass caused by elevation of Pyrenees in the north-east and reopening of strait of Gibraltar in the south (at the end of Messinian Salinity Crisis – 5.33 MYA, Krijgsman *et al.* (1992)).

Expecting high degree of host specificity in *Dactylogyrus*, we used the parasites as an additional tool to reveal historical contacts between cyprinoid hosts in eastern peri-Mediterranean and clarify phylogenetic relationships between endemic cyprinoid species (Paper II). Phylogenetic analyses revealed polyphyletic relationship of *Dactylogyrus* species parasitizing endemic Iberian cyprinids. One group encompassed all *Dactylogyrus* of endemic leuciscids and several species parasitizing on endemic cyprinids, and the second group included *Dactylogyrus* species parasitizing only endemic cyprinids. Interestingly, within the first clade the Iberian species clustered with the non-endemic *Dactylogyrus* parasitizing cyprinoids from other European regions. Therefore, we hypothesized that recent *Dactylogyrus* fauna of the Iberian Peninsula is result of two separated colonization events. The one event is putatively associated with the southern dispersion route of cyprinids (Doadrio, 1990, Perea *et al.*, 2010) from which majority of the Iberian cyprinids originated, together with their host-specific *Dactylogyrus* parasites (i.e. recent species *D. bocageii, D. doadrioi, D. guadianensis, D. lenkoranoïdes,* and *D. mascomai*). The second event is associated with the northern dispersion route via central Europe (Almaça, 1988, Bănărescu, 1992). The paraphyly was revealed also for the group of Moroccan *Dactylogyrus*. Interestingly, *D. marocanus* (the generalist parasite in the North Africa) was revealed to be phylogenetically close to common *Dactylogyrus* species of *Cyprinus carpio* and *Carassius gibelio*. The multiple origin hypothesis is also supported by the basal position of *D. andalousiensis* (endemic species to Iberian Peninsula) to the clade comprising only *Dactylogyrus* parasitizing endemic Moroccan *Luciobarbus*. Additionally, this study highlights potential of *Dactylogyrus* parasites as helpful tool for investigation of phylogeny and phylogeography of their cyprinid hosts.

Species delimitation analysis revealed high number of potentially cryptic species parasitizing endemic *Squalius* spp. and four host genera belonging to *Chondrostoma s.l.* (Paper V). Nine putative species collected from hosts belonging to these two genera share similar morphological features, and are phylogenetically close and morphologically similar to the species parasitizing on congeners from other European areas. This remarkable hidden parasite species diversity is the most likely associated with the adaptive radiation of their leuciscid hosts after their colonization of Iberian Peninsula. Herein, the recent distribution of the individual fish species is usually restricted into single river system and overlapping of the distribution ranges is rare (Doadrio, 1988, Zardoya & Doadrio, 1998, Machordom & Doadrio, 2001, Doadrio *et al.*, 2002, Mesquita *et al.*, 2007, Gante *et al.*, 2015, Casal-López *et al.*, 2017, Sousa-Santos *et al.*, 2019). Therefore, we can assume that parasites co-speciated with their geographically isolated leuciscid hosts. This claim is supported also by observed general congruency between parasite phylogeny revealed in our studies, and leuciscid phylogeny revealed in the previous ichthyological studies (Waap *et al.*, 2011, Sousa-Santos *et al.*, 2019). Nevertheless, the parasite phylogeny does not fully correspond to the phylogeny of their leuciscid hosts. Such minor incongruence may be explained by the more recent, human-induced, secondary contacts of the hosts followed by host switching of parasites.

Section III – Cophylogenetic relationships in Cyprinoidei-*Dactylogyrus* system in peri-Mediterranean

Paper IV

Benovics M., Desdevises Y., Šanda R., Vukić J. & Šimková A. (2020) Cophylogenetic relationships between *Dactylogyrus* (Monogenea) ectoparasites and endemic cyprinoids of the north-eastern European peri-Mediterranean region. *Journal of Zoological Systematics and Evolutionary Research* 58: 1–21. doi: 10.1111/jzs.12341

Paper VII

Benovics M., Vukić J., Šanda R. & Šimková A. (submitted) Disentangling the evolutionary history of peri-Mediterranean cyprinids using host-specific *Dactylogyrus* ecto-parasites (Monogenea: Monopisthocotylea). *Evolution* (January 2020).

As was underlined in the previous two sections, *Dactylogyrus* parasites may serve as good additional marker for investigating historical processes in their hosts. For such purpose cophylogenetic methods based on the comparison of phylogenies of parasites and their hosts may be applied. We used dual-based cophylogenetic approach (distance-based and event-based methods) to assess degree of congruency between host and parasites phylogenies, and estimate which coevolutionary event might play important role in the speciation of parasites within this host-parasite system.

The distance-based analyses revealed highly significant overall cophylogenetic signal between phylogenies of *Dactylogyrus* parasites and endemic cyprinoids (Paper IV). However, in North-Eastern peri-Mediterranean, only ~50% of host-parasite links contributed significantly into global cophylogenetic structure. The majority of statistically significant host-parasite links were inferred in two groups: (1) host-specific *Dactylogyrus* and European representatives of Barbinae subfamily, and (2) generalist *Dactylogyrus* species with unique morphological characters of haptor (*D. alatus*, *D. sphyrna* and *D. vistulae*) and their respective leuciscid hosts. Concluding that cospeciation occurs especially between Barbinae and their host- specific *Dactylogyrus* parasites we focussed primarily on this system (Paper VII).

The Barbinae represent the cyprinid group with the largest distribution range, covering North-west Africa and all southern-European Peninsulas; however, the origin and historical distribution of recent lineages are not fully resolved and different dispersion scenarios were proposed (Bănărescu, 1989, 1992, Doadrio, 1990, Yang *et al.*, 2015). The main dispersion events took place either via river captures in the continental Europe, through North Africa, or are associated with the Messinian Salinity Crisis (5.96 MYA, Krijgsman *et al.* (1992)). Out of the 65 individual host-parasite links between endemic peri-Mediterranean Barbinae and their *Dactylogyrus* parasites, 40 contributed significantly into overall coevolutionary structure. However, the significant links were revealed only between *Barbus*-specific European *Dactylogyrus* species and their respective *Barbus* hosts, and divergent lineage of Iberian *Dactylogyrus* and their respective *Luciobarbus* hosts.

In total, we tested 11 different coevolutionary scenarios to assess significance of the each coevolutionary event in host-parasite system. The event-based cophylogenetic approach revealed that the speciation within *Dactylogyrus* is primarily driven by host switching. Such finding was contrasting to previous assumptions regarding several fish-dactylogyrid systems, where the intra-host duplication was revealed as most frequent cophylogenetic event in congeneric dactylogyrid monogeneans parasitizing fish (Mendlová *et al.*, 2012, Šimková *et al.*, 2004, 2013b). However, it is important to take a note that previous studies included data from either a limited number of host species from investigated area, or phylogenetically distant host species, while in our study the host switches are primarily documented in the case of phylogenetically close fish species with sympatric distribution. Moreover, our results suggest that intra-host parasite duplication occurs frequently also in Cyprinoidei-*Dactylogyrus* system, but only in the instances where the host switching is impossible or highly improbable due to geographic isolation of hosts.

The mapping of morphological characters important for attachment (in this case we selected the shape of haptoral ventral connective bar) into molecular phylogeny validates their taxonomical and phylogenetic importance (Paper VII). Interestingly, the same morphological type of connective bar is present in the endemic North-west African *Dactylogyrus*, *Dactylogyrus* of the Balkan *Luciobarbus* spp., *D. omenti* of Balkan *Aulopyge huegelii,* and two species - *D. carpathicus* and *D. crivellius* parasitizing on southern European *Barbus* spp. However, the molecular phylogeny did not fully resolve relationships between these taxa. The presence of the common morphological element in above mentioned *Dactylogyrus* species supports their common origin. Moreover, the mapping of connective bar into phylogeny suggests that European *Barbus* spp. are parasitized by two evolutionary divergent lineages of *Dactylogyrus* parasites, each of them associated with one of the proposed colonization wave (the northern one via Europe and the southern one via connections between Balkan Peninsula, Anatolia, and North Africa (Doadrio, 1990)).

Hence, we conclude that *Dactylogyrus*-Cyprinoidei represent unique system where the host-parasite cospeciation is also quite frequent in the phylogenetically divergent host lineages;

however, the host switching of parasites plays the main role in *Dactylogyrus* diversification allowing some *Dactylogyrus* species parasitize wide range of hosts. Therefore, we can observe different levels of host specificity in *Dactylogyrus,* ranging from strict specialists, throughout intermediate specialists, to true generalists. We also proposed that the evaluation of morphological characters (especially shape of haptoral elements) in the phylogenetic studies may help us to resolve the uncertain phylogenetic relationships between *Dactylogyrus* lineages.

6 Conclusion and future perspectives

As mentioned above, *Dactylogyrus* parasites are almost exclusively host-specific to cyprinoid fishes (approximately 95%, see Gibson *et al.* (1996) for non-cyprinoid records). Up to this date their presence was reported from species of eight out of twelve cyprinoid families (except Paedocypridae, Psilorhynchidae, Sundadanionidae, and Tanichthyidae). However, the reports of *Dactylogyrus* is the most likely biased by the lack of parasitological research on abovementioned families (see host-parasite lists compiled by Gibson *et al.* (1996) and in Pugachev *et al.* (2009)).

The species diversity of *Dactylogyrus* in the peri-Mediterranean was revealed to be remarkably high and underexplored; however, *Dactylogyrus* communities are species poorer in comparison to Central Europe or Asia. Each of the endemic cyprinoid host species in the peri-Mediterranean area is parasitized by comparatively lower number of *Dactylogyrus* species when compared to the widely distributed European cyprinoid hosts, which was expected considering geographic isolation of peri-Mediterranean endemics and their highly limited distribution range. Phylogenetically related endemic cyprinoids often harbor morphologically similar *Dactylogyrus* species, making the species identification rather difficult. However, molecular analyses revealed, that some fish species harbour host-specific *Dactylogyrus* species which are actually cryptic or pseudocryptic (morphologically similar and almost undistinguishable, but different on molecular level) (Papers III, V, VI). So far, we described eight new species from the Euro-Mediterranean region (Papers I, VI); however, other species will be described in near future (i.e. *Dactylogyrus* from Iberian Peninsula, see Paper V). The Afro-Mediterranean appears to be also underexplored in regards to *Dactylogyrus* fauna. Four new species were described recently (Rahmouni *et al.*, 2017); however, considering recent taxonomical research on the cyprinid hosts (especially rapidly evolving *Luciobarbus*) in this region (Touil *et al.*, 2019), cryptic diversity of *Dactylogyrus* may be also expected. Relatively high number of host specific species was revealed among investigated peri-Mediterranean *Dactylogyrus*. Out of 91 species collected from 127 cyprinoid host species 38% were strict specialists parasitizing only single cyprinoid species. Moreover, 44% species were recorded as intermediate generalists parasitizing on congeneric hosts.

Šimková *et al.* (2004) showed that intra-host duplication plays the most important role in the speciation of *Dactylogyrus* parasitizing central European cyprinoids. Initial assumption of intra-host duplication in *Dactylogyrus* was based on the presence of several morphologically similar species on the single host species. Later, using the same dataset of *Dactylogyrus* parasites and their hosts, Miguel-Lozano *et al.* (2017) supported that duplication is far more frequent in this system in comparison to two other monogenean-fish host systems (i.e. *Gyrodactylus* and *Lamellodiscus*, and their respective hosts); however, their analyses also revealed exceptionally high number of "sorting events" (i.e. „loss and failure to diverge"). Based on the further investigation, the most prevalent coevolutionary event in the speciation of *Dactylogyrus* actually appears to be host switching (Míguez-Lozano *et al.*, 2017, Paper IV, Paper VII). Host switching is promoted in the case of fish hosts living in sympatry. In general, the strong coevolutionary structure was revealed in the *Dactylogyrus*-Cyprinoidei system. Nevertheless, significant coevolutionary links were detected only between cyprinids (i.e. *A. huegelii*, *Barbus* spp. and *Luciobarbus* spp.) and their host-specific *Dactylogyrus* parasites (Paper IV). Therefore, we focused specifically on the cophylogenetic relationships in this system included majority of peri-Mediterranean species belonging to Cyprinidae (representatives of Barbinae) and their host-specific *Dactylogyrus* species (Paper VII). Our results suggested that *Dactylogyrus* species specialized on their European *Barbus* and *Luciobarbus* hosts due to the separated dispersion routes of these Barbinae. Moreover, several parasites switched to phylogenetically distant host species (i.e. *Luciobarbus* and *Carasobarbus*) which was facilitated by the formation of the land-bridges between Europe and Africa.

Unfortunately, to clarify the coevolutionary pattern of *Dactylogyrus*-cyprinoids in a whole range of peri-Mediterranean and to reveal the patterns of historical biogeography of cyprinids using host specific *Dactylogyrus*, there are still gaps in the molecular data for *Dactylogyrus* species and their cyprinoid hosts in some areas. As was proposed in the Paper I, the Balkan endemic *Dactylogyrus* species, parasitizing on the ancestral cyprinid lineages morphologically resemble species endemic in the Middle East; however, the molecular data from this region are still missing. Nevertheless, the diversity of *Dactylogyrus* parasites in Middle East was well explored (see numerous checklists focusing on the specific subregions compiled by Öktener (2003), Abdullah *et al.* (2004), Selver *et al.* (2009), Neary *et al.* (2012), Pazooki & Masoumian (2012), Soylu (2012), Aydoğdu *et al.* (2015), Aydoğdu & Kubilay (2017), Mhaisen & Abdullah (2017), Mhaisen & Abdul-Ameer (2019)), but determination of parasite species is often incomplete and some species are listed ambiguously (i.e. see *Dactylogyrus* spp. in Mhaisen & Abdul-Ameer (2019)). Therefore, more thorough investigation in this region, using integrative taxonomical approach is necessary. Moreover, considering phylogenetic relatedness of *Luciobarbus* spp. to Middle Eastern genus *Capoeta*, the molecular data of *Dactylogyrus* of *Capoeta* might fill gaps in the cophylogenetic relationships between cyprinids and their host-specific *Dactylogyrus* parasites (as suggested in Paper VII). A single study including molecular characterization of *Dactylogyrus* species was published from Middle

East; however, this study focused on the *Dactylogyrus* of cyprinids introduced to Iran i.e. *Cyprinus carpio* L., and *Ctenopharyngodon idella* (Valenciennes, 1844) (Ahmadi *et al.*, 2017).

The next step in investigating evolutionary relationships among *Dactylogyrus*, and cophylogenetic relationships with their cyprinoid hosts, might represent phylogenomic approach. Up to this date, the full genomic data on the monogeneans are limited and exist for only two species – *Gyrodactylus salaris* (Hahn *et al.*, 2014), and *Protopolystoma xenopodis* (Price, 1943) (not published yet, accession BioProject number PRJEB1201). Therefore, it may be rather difficult to assembly complete genomes from dactylogyrid monogeneans. Nevertheless, multiple complete mitogenomes were recently published for species of *Gyrodactylus* (e.g Huyse *et al.*, 2007, Plaisance *et al.*, 2007 Bachmann *et al.*, 2016, Zhang *et al.*, 2016, Vanhove *et al.*, 2018), *Cichlidogyrus* (Vanhove *et al.*, 2018), *Benedenia* Diesing, 1858 (Antonio Baeza *et al.*, 2019), *Lamellodiscus* (Zhang *et al.*, 2018a), *Lepidotrema* Johnston & Tiegs, 1922 (Zhang *et al.*, 2018a), *Tetrancistrum* Goto & Kikuchi, 1917 (Zhang *et al.*, 2014), three diplozoid species (Zhang *et al.*, 2018b), and single *Dactylogyrus* species – *D. lamellatus* Achmerow, 1952 (Zhang *et al.*, 2018c). Provided mitochondrial genomes may serve as optimal templates for assembling mitogenomes of more monogenean species, especially in highly diversified *Dactylogyrus*. However, the main issue remains with the collection of optimal material for NGS (Next Generation Sequencing). As the quantity and quality of the genomic DNA isolated from single worm may be not sufficient enough, one of the option represents pooling of considerable number of specimens of one species (Vanhove *et al.*, 2018). However, considering that monogeneans are generally small sized and correct species identification is rather difficult without magnifying optic methods, there is high risk of cross-species contamination in the pooled samples rendering the obtained genomic data difficult to process.

As was tackled in Paper III, *Dactylogyrus* parasites may represent suitable organism for the population genetic studies. This concerns especially generalist species with exceptionally wide distribution range, such as *D. vistulae* or *D. vastator* (Moravec, 2001, Paper III). These species are morphologically unique and may be easily distinguished from other *Dactylogyrus* species due to their relatively large body size and enlarged haptoral elements (Pugachev *et al.*, 2009) using only stereomicroscope. Nonetheless, a priori development of more suitable genetic markers for population genetics is necessary. The suitable methods could potentially represent analysis of microsatellite loci (short tandem repeats) or RAD-sequencing (restriction associated DNA markers). The population genetics might uncover patterns of recent distribution of widely distributed generalist species, their origin and potentially their risk of introduction into non-native regions.

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Appendix

Full texts of original research papers representing results of the Ph.D. thesis are included as appendices. Papers are ordered chronologically by publication or submission date.

PAPER I

The phylogenetic position of the enigmatic Balkan *Aulopyge huegelii* (Teleostei: Cyprinidae) from the perspective of host-specific *Dactylogyrus* parasites (Monogenea) with a description of *Dactylogyrus omenti* n. sp.

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Parasites & Vectors

RESEARCH

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The phylogenetic position of the enigmatic Balkan *Aulopyge huegelii* (Teleostei: Cyprinidae) from the perspective of host-specific *Dactylogyrus* parasites (Monogenea), with a description of *Dactylogyrus omenti* n. sp.

Michal Benovics*, Maria Lujza Kičinjaová and Andrea Šimková

Abstract

Background: The host specificity of fish parasites is considered a useful parasite characteristic with respect to understanding the biogeography of their fish hosts. *Dactylogyrus* Diesing, 1850 (Monogenea) includes common parasites of cyprinids exhibiting different degrees of host specificity, i.e. from strict specialism to generalism. The phylogenetic relationships and historical dispersions of several cyprinid lineages, including *Aulopyge huegelii* Heckel, 1843, are still unclear. Therefore, the aims of our study were to investigate (i) the *Dactylogyrus* spp. parasites of *A. huegelii*, and (ii) the phylogenetic relationships of *Dactylogyrus* spp. parasitizing *A. huegelii* as a possible tool for understanding the phylogenetic position of this fish species within the Cyprininae lineage.

Results: Two species of *Dactylogyrus*, *D. vastator* Nybelin, 1924 and *D. omenti* n. sp., were collected from 14 specimens of *A. huegelii* from the Šujica River (Bosnia and Herzegovina). While *D. vastator* is a typical species parasitizing *Carassius* spp. and *Cyprinus carpio* L, *D. omenti* n. sp. is, according to phylogenetic reconstruction, closely related to *Dactylogyrus* species infecting European species of *Barbus* and *Luciobarbus*. The genetic distance revealed that the sequence for *D. vastator* from *A. huegelii* is identical with that for *D. vastator* from *Barbus plebejus* Bonaparte, 1839 (Italy) and *Carassius gibelio* (Bloch, 1782) (Croatia). *Dactylogyrus omenti* n. sp. was described as a species new to science.

Conclusions: Our findings support the phylogenetic position of *A. huegelii* within the Cyprininae lineage and suggest that *A. huegelii* is phylogenetically closely related to *Barbus* and *Luciobarbus* species. The morphological similarity between *D. omenti* n. sp. and *Dactylogyrus* species of Middle Eastern *Barbus* suggests historical contact between cyprinid species recently living in allopatry and the possible diversification of *A. huegelii* from a common ancestor in this area. On other hand, the genetic similarity between *D. vastator* ex *A. huegelii* and *D. vastator* ex *C. gibelio* collected in Balkan Peninsula suggests that *A. huegelii* was secondarily parasitized by *D. vastator*, originating from *C. gibelio* after introduction of this fish species from Asia to Europe.

Keywords: Host specificity, Coevolution, Phylogeography, *Aulopyge*, Cyprininae, *Dactylogyrus*

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* Correspondence: michal.benovics@gmail.com
Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic

Background

Parasites and their hosts are usually closely associated due to their coevolution, realized by reciprocal genetic adaptations between these interacting species. In evolutionary time, this leads to a selection for improvements in host–parasite recognition mechanisms [1]. The high degree of host specificity among parasites (generally, a parasite species is restricted to a single host species), reflecting parasite specialization, may arise from such coevolutionary interactions [2–4] In the case of high host specificity, the phylogeny of host-specific parasites may even follow the phylogeny and historical biogeography of their hosts as a result of co-speciation [5, 6]. However, parasite diversification can also be driven by host specialization following host switching resulting from strong ecological association, as was shown for monogeneans of marine fish [7]. The host specificity of freshwater fish parasites appears to be a useful characteristic in terms of understanding the biogeography of freshwater fishes (e.g. [8–11]). Basic host specificity is commonly expressed by the number of host species (also termed host range). However, other aspects, like the ecological performance of the parasite, the phylogenetic affinities of hosts, and the biogeographical distribution of the parasite, are important when expressing host specificity [12].

Gill ectoparasites of the genus *Dactylogyrus* Diesing, 1850 generally exhibit a high degree of host specificity and a high species diversity arising from the multitude of cyprinid fish species, which are common hosts of these parasite species [13]. Šimková et al. [14] defined several levels of host specificity for *Dactylogyrus* using an index of host specificity, expressed as the inverted value of the index of non-specificity proposed by Desdevises et al. [7]. Five *Dactylogyrus* groups were defined ranging from strict specialists, which occur on a single host species, to true generalists, which parasitize different, phylogenetically unrelated cyprinid host species. These host-specific parasites have a direct life-cycle, in which the larval stage (oncomiracidium) actively searches for suitable host species, using chemical cues for host recognition [15]. Therefore, among monogeneans, a high degree of adaptation to their host resource is required [16–19]. Several studies documented microhabitat restriction (i.e. preferred niche measured by specific gill positions) in *Dactylogyrus* species [20–24]. Since different parts of gills offer different types of substrate, niche preference is associated with a specific type and shape of attachment organ (haptor) in parasites assigned to *Dactylogyrus* [9, 21, 23]. Šimková et al. [23] also revealed that there is morphological adaptation of the haptor in species that specifically parasitize phylogenetically related hosts, such as *Dactylogyrus* species parasitizing *Cyprinus carpio* L. and *Carassius auratus* L. of the subfamily Cyprininae. The phylogeny of highly host-specific *Dactylogyrus* species reflects the biogeography and evolutionary history of their cyprinid hosts [25]. Besides some accidental infections of unsuitable hosts, the sharing of *Dactylogyrus* species among evolutionary divergent cyprinid species living in sympatry is rare [23].

The cyprinid fauna of the Balkan Peninsula is extremely rich in endemic species [26]. According to Oikonomou et al. [27], the Balkan freshwater fish fauna represents 59% of all known cyprinid species. The ancient Dessaretes lake system played an important role in cyprinid speciation, which originated during the Pleistocene and is considered as a hotspot of endemic freshwater biodiversity [28–32]. Presently, all the great lakes in the Balkan Peninsula, the Ohrid, Prespa, Mikri Prespa and Maliq lakes (the latter one was drained after World War II), are parts of this system. Albrecht & Wilke [30] also theorized that during the Miocene and Pliocene eras the whole Dessaretes basin was filled with water and that all lakes were connected. After the closing of the Korca Depression and connections between the Dessaretes and the Paratethys, the water level decreased and fragmentation of the populations triggered allopatric speciation, which led to rich freshwater fish diversity. Zardoya et al. [33] investigated the geographical origin of Balkan endemic cyprinids. They suggested that cyprinid fauna colonized the Balkan Peninsula during two different time periods. The first wave occurred during the Miocene and the second during the Plio-Pleistocene via river captures. The phylogenetic relationships among Balkan cyprinid taxa and their biogeographical histories have been actively studied over the last 25 years (e.g. [34–40]). Studying host-specific parasites, such as *Dactylogyrus*, may represent an additional tool for investigation and may shed more light on both the historical contacts between cyprinid hosts and their phylogeography.

The Dalmatian barbelgudgeon (*Aulopyge huegelii* Heckel, 1843), the only representative of the monotypic genus *Aulopyge*, is one of the many endangered cyprinid species of the Balkan Peninsula. Its distribution is limited to the Dinaric karst rivers and lakes of Croatia and Bosnia and Herzegovina [41–43]. Although previously quite abundant, in recent years *A. huegelii* populations have been declining [44]. Tsigenopoulos & Berrebi [43] considered the ancestor of *A. huegelii* as the first migration wave of cyprinids to the Mediterranean region, which found refuge in Dalmatia. According to the molecular clock, they estimated that European *Barbus* and *A. huegelii* diverged during the middle Miocene, which concurs with the first wave colonizing Balkan Peninsula [33]. On the basis of mitochondrial cytochrome *b* sequence data, Tsingenopoulos et al. [45] suggested that *A. huegelii* is the sister clade to the clade including *Barbus* + *Luciobarbus* lineages. However, Yang et al. [46] showed that *Aulopyge* is the sister taxon to the the European *Barbus* lineage, well separated from the *Luciobarbus* lineage, and, according to Wang et al. [47], the

European *Barbus* (sensu stricto) lineage and *A. huegelii* share a common ancestor (originating in the Qinghai-Tibetan Plateau region about 19.4–7.8 Mya) with the species of the Asian genera *Schizothorax* and *Cyprinion*.

Until now, only a very few endemic cyprinid species from the Balkan Peninsula have been investigated for parasites [48–54]. As previously shown by Šimková et al. [25], phylogenetic relationships between *Dactylogyrus* lineages can reflect cyprinid phylogeny. Thus, we hypothesized that the phylogenetic relationships between host-specific *Dactylogyrus* species of *A. huegelii* and those parasitizing other closely related cyprinid species will support the phylogenetic position of this monotypic cyprinid genus. Therefore, the aims of our study were (i) to investigate the *Dactylogyrus* fauna of endemic *A. huegelii*, and (ii) to investigate the phylogenetic relationships between *Dactylogyrus* species parasitizing *A. huegelii* and those parasitizing species of the Cyprininae distributed in Europe, i.e. *Barbus* spp., *Carassius* spp. and *C. carpio* (the last two originating from Asia and widely distributed throughout the whole of Europe). As a result, we described a new species of *Dactylogyrus* collected from endemic *A. huegelii*.

Methods

Sampling and species identification

A total of 14 specimens of *Aulopyge huegelii* from the Šujica River, Bosnia and Herzegovina, were sampled in July 2015. Fish were dissected using standard methods [55]. *Dactylogyrus* specimens were collected from host gills, fins, head surfaces, and oral and nasal cavities, mounted on slides and covered with a mixture of glycerine and ammonium picrate (GAP [56]) for further identification. The identification of monogeneans was performed using Gussev [57] on the basis of the size and shape of the hard parts of the attachment organ, the haptor, and the reproductive organs which represent species-specific morphological characters. Identification to species level was performed using an Olympus BX51 microscope equipped with phase contrast optics. Several *Dactylogyrus* specimens were bisected; one half of the body (usually the half with the reproductive organs) was mounted on a slide for species identification, the other was individually preserved in 96% ethanol for DNA extraction. Basic epidemiological data, i.e. prevalence, mean abundance, minimum and maximum intensity of infection, were calculated for each species according to Bush et al. [58]. Prevalence, as the percentage of fish infected by a given parasite species, and mean abundance, as the mean number of parasite specimens per individual host taking into account both infected and uninfected hosts, were calculated.

Morphometric data

Morphometric measurements of *Dactylogyrus* spp. specimens (modified according to Gussev [57]) were taken using Digital Image Analysis (Stream Motion). All measurements of morphometric characters are in micrometres and are presented as the range followed by the mean and the number of measured specimens (*n*) in parentheses. The numbering of marginal hook pairs for *Dactylogyrus* follows the recommendations by Mizzele [59]. After measuring morphometric characters, the specimens were removed from GAP and remounted in Canada balsam, according to Ergens [60], and deposited as type-specimens in the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, in České Budějovice (IPCAS).

DNA extraction, amplification and sequencing

Parasites were removed from storage ethanol and dried by means of a vacuum centrifuge. DNA extraction was performed using a standard protocol (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Partial 18S rDNA and entire ITS1 regions were amplified using primers S1 (5′-ATT CCG ATA ACG AAC GAG ACT-3′) and IR8 (5′-GCT AGC TGC GTT CTT CAT CGA-3′) [61], which anneal to the 18S and 5.8S rDNA regions, respectively. Each amplification reaction for partial the 18S rDNA and ITS1 regions was performed in a final volume of 15 μl, containing 0.3 μl of Taq polymerase, 1.5 μl buffer, 0.9 μl $MgCl_2$, 0.3 μl of dNTPs, 1.5 μl of each primer and 2.5 μl of pure DNA (20 ng/μl). PCR was carried out using the following steps: 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 53 °C and 90 s at 72 °C, and 10 min of final elongation at 72 °C. Partial 28S rDNA was amplified using the forward primer C1 (5′-ACC CGC TGA ATT TAA GCA-3′) and the reverse primer D2 (5′-TGG TCC GTG TTT CAA GAC-3′) [62]. PCR followed the protocol included in Šimková et al. [14]. PCR products were checked on 1.5% agarose gels, purified by using an ExoSAP-IT kit (Ecoli, Bratislava, Slovakia), following the manufacturer's protocol, and sequenced directly using the PCR primers and BigDye Terminator Cycle sequencing kit (Applied Biosystems, Pardubice, Czech Republic). Sequencing was carried out using an ABI 3130 Genetic Analyzer (Applied Biosystems). The newly generated sequences were deposited in the GenBank database and molecular vouchers (hologenophores, paragenophores [63]) were deposited in the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, in České Budějovice (IPCAS).

Phylogenetic analyses

DNA sequences were aligned using fast Fourier transform in MAFFT [64]. To match the lengths of the newly obtained sequences to the sequences obtained from GenBank, they were optimized manually. A test of homogeneity to examine the congruence of two datasets (partial 18S with the ITS1 region vs 28S rDNA) was performed in PAUP* 4b10 [65]. Since the difference was not statistically significant ($P = 0.737$), the concatenated data were used for further phylogenetic analyses. The sequences of *Dactylogyrus extensus* Mueller & Van Cleave, 1932 parasitizing *C. carpio* were acquired from GenBank (accession numbers KM277459 and AY553629 for partial 18S rDNA with the ITS1 region and partial 28S rDNA sequences, respectively). The sequences of partial 18S rDNA with the ITS1 region and partial 28S rDNA for *Dactylogyrus vastator* Nybelin, 1924 and *Dactylogyrus anchoratus* (Dujardin, 1845) parasitizing *Carassius gibelio* (Bloch, 1782), and *Dactylogyrus* species parasitizing *Barbus barbus* L., *B. balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002, *B. prespensis* Karaman, 1924, *Luciobarbus graecus* (Steindachner, 1895) and *L. albanicus* (Steindachner, 1870) (including Balkan endemic and non-endemic *Dactylogyrus* species) were included in phylogenetic analyses due to the proposed evolutionary proximity of the host species. The final tree was rooted using *Dactylogyrus* species of *C. gibelio* and *C. carpio* as the outgroup taxa, following Šimková et al. [23].

To analyze the genetic distances between the specimens of *D. vastator* from different host species, sequences of partial 18S rDNA and complete ITS1 available for *D. vastator* were obtained from GenBank. The uncorrected p-distances between *D. vastator* collected from 5 different host species from 7 localities were calculated using MEGA6 [66].

Gaps and ambiguously aligned regions were removed from the alignment using GBlocks v. 0.91 [67]. The most appropriate DNA evolution model was determined using the Akaike information criterion (AIC) in JModelTest 2.1.10 [68, 69]. Phylogenetic trees were inferred by Bayesian inference (BI) and Maximum Likelihood (ML) analyses using MrBayes 3.2 [70] and PhyML 3.0 [71], respectively. The search for the best ML tree was performed using NNI (nearest neighbour interchange) and SPR (subtree pruning and regrafting) branch swapping algorithms with six substitution categories. The clade support for ML was assessed by 1000 bootstrap pseudoreplicates. Bayesian inference trees were constructed using the MC^3 algorithm with two parallel runs containing one cold and three hot chains. The analysis ran for 10^7 generations and tree topologies were sampled every 100 generations. The first 25% of all saved trees were discarded as relative ‘burn-in’ periods according to standard deviation split frequencies (< 0.01). Posterior probabilities were calculated as the frequency of samples recovering any particular clade.

Results

Parasites of *A. huegelii*

All 14 dissected fish specimens were infected with monogenean parasites. *Dactylogyrus* spp. reached 93% prevalence in *A. huegelii* and represented two species. The first was *Dactylogyrus vastator*, a common parasite of *Carassius* spp. and *C. carpio*, and which also accidentaly infects some other fish species ([13], M. Benovics, unpublished data). Morphological identification confirmed that specimens of *D. vastator* from *A. huegelii* possess the same morphology of the hard parts of the haptor and reproductive organs (i.e. the shape was identical and the size of these parts was within the range of sizes included in original description of *D. vastator*). The second species is here described as *Dactylogyrus omenti* n. sp., which was not found on other endemic *Barbus* species, or any other cyprinids collected in the Balkan Peninsula, and is most likely specific to *A. huegelii*. Both *Dactylogyrus* species differed in their epidemiological characteristics (Table 1). The prevalence of *D. omenti* n. sp. was significantly higher than that of *D. vastator* (Fisher's exact test, $P = 0.006$, $df = 1$). The abundance of *D. omenti* n. sp. was higher than that of *D. vastator* (Mann-Whitney test, $U_{(14)} = 15.00$, $Z = 3.79$, $P < 0.001$).

Phylogenetic position of *Dactylogyrus* spp. parasitizing *A. huegelii*

A final concatenated sequence alignment was constructed using 1625 unambiguously aligned nucleotide positions. GTR + I + G was selected as the optimal evolution model. ML and BI analyses provided phylogenetic trees with similar topologies. The BI tree is presented in Fig. 1, where bootstrap values resulting from ML analysis and posterior probabilities resulting from BI analysis are included. Collection localities and GenBank accession numbers of all newly generated sequences used in the phylogenetic reconstructions are provided in Table 2.

The resulting tree for *Dactylogyrus* spp. supports the close phylogenetic relationship of *A. huegelii* to endemic Mediterranean *Barbus* and *Luciobarbus* species and to the widely distributed European *Barbus barbus*, as previously shown by molecular phylogenetic studies of cyprinid fishes [43, 45, 47, 72], i.e. *Dactylogyrus omenti* n. sp. from *A. huegelii* was nested within *Dactylogyrus* spp. from *Barbus* species. *Dactylogyrus vastator* clustered with *D. extensus* from *C. carpio* and with *D. anchoratus* from *C. gibelio*. This clade was well separated from the clade of *Dactylogyrus* species parasitizing *Barbus*, *Luciobarbus* and *A. huegelii*. By comparing the genetic distances of *D. vastator* specimens from different hosts using the sequences of partial 18S and the ITS1 regions (Table 3), we conclude that *D. vastator* from *A. huegelii* is genetically identical to *D. vastator* collected from *C. gibelio* from Croatia and *Barbus*

Table 1 Basic epidemiological data for *Dactylogyrus* species collected from *A. huegelii*

Species	P (%)	MA	I
D. vastator	29	0.3	1
D. omenti n. sp.	93	3.4	1–8

Abbreviations: *P*, prevalence; *MA*, mean abundance, *I*, intensity of infection

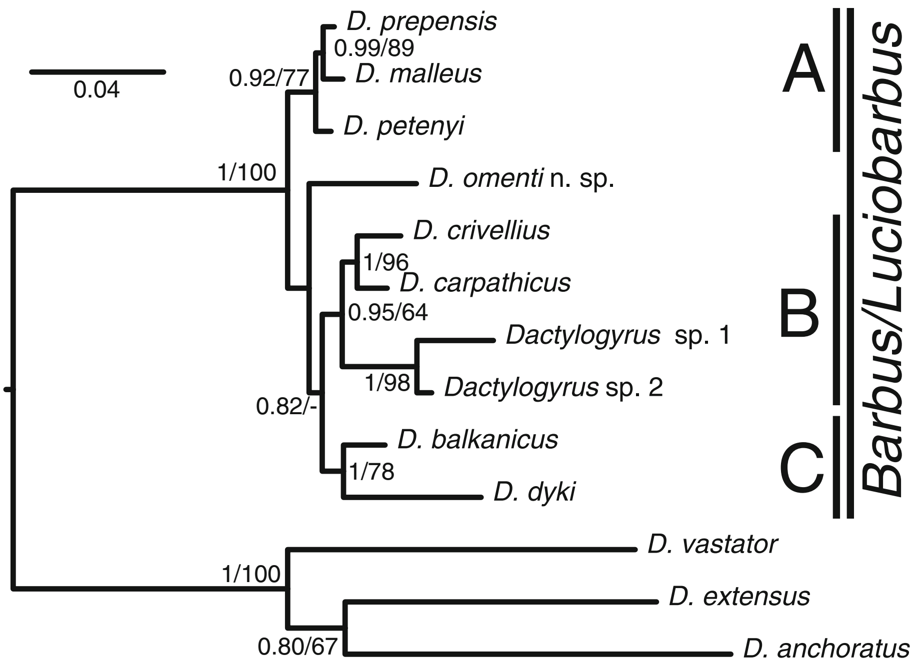

Fig. 1 Phylogenetic tree constructed by Bayesian Inference (BI) analysis. The tree is based on concatenated data of partial 18S and ITS1 rDNA sequences with partial 28S rDNA sequences for selected *Dactylogyrus* species. Values along branches indicate BI posterior probabilities and Maximum Likelihood (ML) bootstrap values as BI/ML. Values < 0.80 for BI and < 50% for ML are indicated by dashes or not shown. Length of branches corresponds to the expected number of substitutions per site. Groups A, B and C refer to the different lineages of *Dactylogyrus* species parasitizing European *Barbus* and *Luciobarbus* species

plebejus Bonaparte, 1839 from Italy. In comparison to species collected in central Europe and eastern Asia, *D. vastator* from *A. huegelii* is closer to *D. vastator* of *C. carpio* (p-distance = 0.003) than to *D. vastator* of *C. gibelio* from the Czech Republic or to *D. vastator* of *C. auratus* from China (p-distance > 0.043).

Dactylogyrus species recovered from *Barbus* spp. formed a paraphyletic group with the nested position of *Dactylogyrus* spp. from Greek *Luciobarbus* and *D. omenti* n. sp. Three well- (or moderately-) supported groups were recognized for *Dactylogyrus* species collected from *Barbus* and *Luciobarbus* hosts (Fig. 1). Group A (PP = 0.92, BS = 77) comprised *D. prespensis* Dupont & Lambert, 1986, *D. malleus* Linstow, 1877 and *D. petenyi* Kastak, 1957, which exhibit a similar shape of the male copulatory organ (MCO). Group B was formed by two well supported clades, the first including *D. carpathicus* Zachvatkin, 1951 and *D. crivellius* Dupont & Lambert, 1986 collected from *Barbus*, and the second including two undescribed species *Dactylogyrus* sp. 1 and *Dactylogyrus* sp. 2 collected from Greek *Luciobarbus*. All these species exhibit a similar shape of the haptoral hard parts, especially in having a cross-shaped connective ventral bar with 5 marginal extremities, but differ between clades in the shape of the MCO. The last supported grouping (group C in Fig. 1, PP = 1, BS = 78) comprised *D. balkanicus* Dupont & Lambert, 1986 and *D. dyki* Ergens & Lucky, 1959. While *D. dyki* is a widely distributed European species (i.e. infecting a wide range of *Barbus* spp.), *D. balkanicus* appears to be endemic to the Balkan Peninsula, and they both share a similar shape of the MCO. *Dactylogyrus omenti* n. sp. was found at the basal position in the group of *Dactylogyrus* species parasitizing *Barbus* and *Luciobarbus*. However, the phylogenetic position of *D. omenti* n. sp. in relation to *Dactylogyrus* groups A, B and C was not resolved.

Family Dactylogyridae Bychowsky, 1933
Genus Dactylogyrus Diesing, 1850

Dactylogyrus omenti n. sp.

***Type-host*:** *Aulopyge huegelii* Heckel, 1843 (Cypriniformes: Cyprinidae).
***Type-locality*:** Locality Duvansko polje, River Šujica, Bosnia and Herzegovina (43°42′05.7″N, 17°15′50.5″E).
***Type-material*:** The holotype, 4 paratypes, 1 hologenophore and 3 paragenophores are deposited under the accession number IPCAS M-629.
***Site on host*:** Gill lamellae.
***Representative DNA sequences*:** A nucleotide sequence of partial 28S rDNA (791 bp long; KY201105) and nucleotide sequences representing a fragment (939 bp long;

Table 2 List of newly obtained *Dactylogyrus* species used for molecular analyses and phylogenetic reconstruction

Host species	*Dactylogyrus* spp.	Country	Locality	Coordinates	GenBank accession numbers	
					18S rDNA + ITS1 + 5.8S rDNA	28S rDNA
Aulopyge huegelii	*D. omenti* n. sp.	Bosnia and Herzegovina	Šujica, Duvansko polje	43°42′05.7″N, 17°15′50.5″E	KY201091	KY201105
	D. vastator				KY201092	KY201106
Barbus balcanicus	*D. petenyi*	Greece	Vardar, Axiopolis	40°59′28.4″N, 22°33′14.5″E	KY201097	KY201113
Barbus barbus	*D. carpathicus*	Czech Republic	River Svratka	49°05′32.1″N, 16°37′11.0″E	KY201098	KY201111
	D. malleus				KY201099	KY201112
Barbus prespensis	*D. crivellius*	Albania	Shkumbini, Perrenjas	41°03′50.9″N, 20°33′56.6″E	KY201094	KY201108
	D. prespensis				KY201096	KY201110
	D. balkanicus				KY201093	KY201107
	D. dyki	Greece	Aoos, Kalithea	40°01′16.7″N, 20°41′40.2″E	KY859804	KY859803
Barbus plebejus	*D. vastator*	Italy	River Po	na	KY201104	na
Carassius gibelio	*D. vastator*	Czech Republic	River Dyje	48°48′09.4″N, 16°50′19.3″E	KY201103	na
	D. anchoratus				KY859795	KY863555
	D. vastator	Croatia	Baštica Reservoir	44°11′34.1″N, 15°24′40.7″E	KY207446	na
Luciobarbus albanicus	*Dactylogyrus* sp. 1	Greece	Lake Trichonis, Panetolio	38°35′20.2″N, 21°28′02.7″E	KY201100	KY201114
Luciobarbus graecus	*Dactylogyrus* sp. 2	Greece	Sperchios, Ypati	38°54′14.3″N, 22°17′30.2″E	KY201101	KY201115

Abbreviation: *na*, not available

Table 3 Uncorrected pairwise distances between sequences for *D. vastator* collected from *Aulopyge huegelii* and different species of Cyprininae

Host species[a]		Locality	2	3	4	5	6	7
1	*Aulopyge huegelii* (KY201091)	River Šujica, Bosnia and Herzegovina	0.000	0.001	0.042	0.042	0.047	0.004
2	*Barbus plebejus* (KY201104)	River Po, Italy		0.001	0.042	0.042	0.047	0.004
3	*Carassius gibelio* (KY207446)	Baštica, Croatia			0.043	0.043	0.048	0.003
4	*Carassius gibelio* (KY201103)	River Dyje, Czech Republic				0.002	0.004	0.047
5	*Carassius auratus* (KJ854363)	Nanyang, Henan, China					0.004	0.047
6	*Carassius auratus* (KM487695)	River Ergis, China						0.051
7	*Cyprinus carpio* (AJ564159)	River Morava, Czech Republic						

[a]GenBank accession numbers included

Genetic distances were calculated using the sequences of partial 18S rDNA and ITS1 (see Table 2 for accession numbers for *D. vastator* sequences generated in this study)

KY201091) including partial 18S rDNA (446 bp), the ITS1 region (493 bp) and 5.8S (6 bp). No intraspecific variability was found (6 specimens were analyzed).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN) [73], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:723FC725-1C88-4DF6-8ECE-ADC1EE658F8B. The LSID for the new name *Dactylogyrus omenti* is urn:lsid:zoobank.org:act:697DD685-1B87-4FB4-B3CA-65000EC772FF.

Etymology: The specific name is derived from Latin (*omentum* = membrane, bowels) and refers to the shape of the accessory piece.

Description

[Based on 13 specimens in GAP; Figs. 2 and 3.] Body length 230–522 (362; *n* = 3), with greatest width 57–128 (95; *n* = 3), usually near mid-length. One pair of anchors

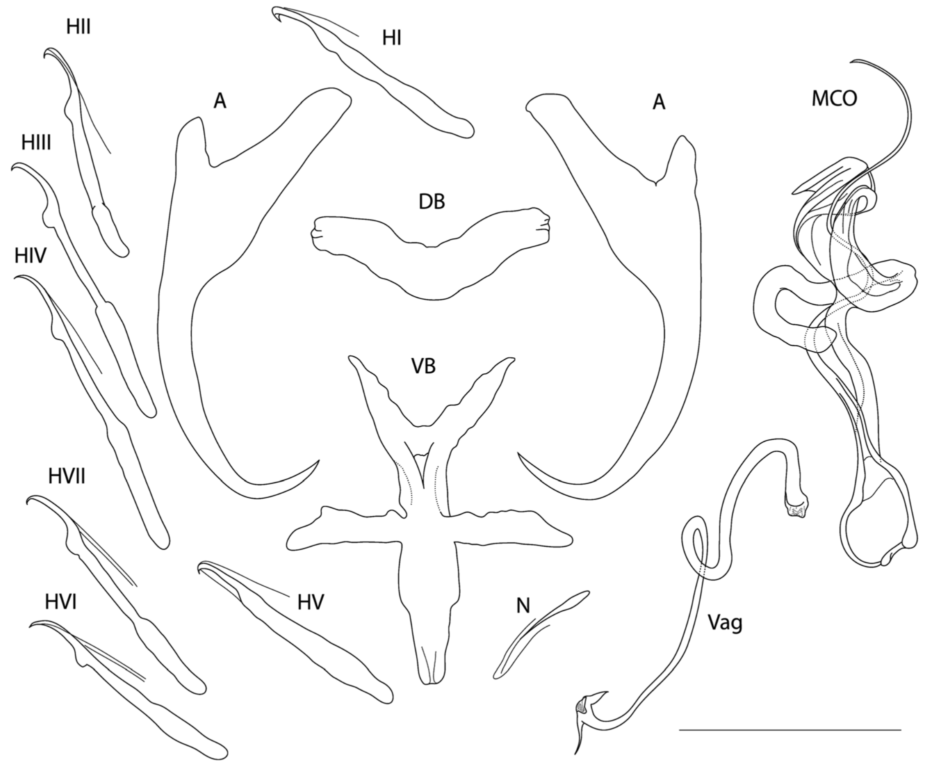

Fig. 2 Drawings of hard parts of haptor and reproductive organs of *Dactylogyrus omenti* n. sp. *Abbreviations*: A, anchors; DB, dorsal connective bar; VB, ventral connective bar; H, marginal hooks (pairs I–VII); N, needle; MCO, male copulatory organ; Vag, vagina. *Scale-bar*: 20 μm

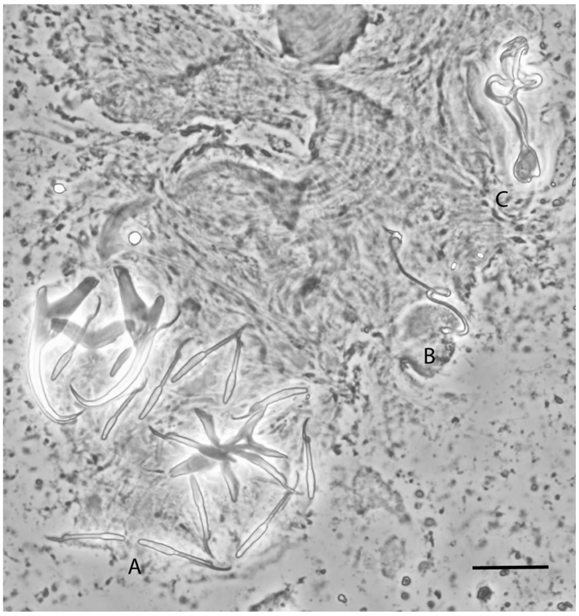

Fig. 3 Phase contrast photomicrograph of hard parts of *Dactylogyrus omenti* n. sp. *Abbreviations*: A, haptor; B, vagina; C, male copulatory organ. *Scale-bar*: 20 μm

(dorsal), inner length 37–41 (38; *n* = 10), outer length 34–37 (35; *n* = 10). Inner root long, extending to broader base in its medial part, 11–16 (14; *n* = 10); outer root short, slightly pointed outward, 3–6 (5; *n* = 10), with moderately curved shaft and short turned-in point, 6–7 (6; *n* = 10). Dorsal bar saddle-shaped, with subterminal folding, total length 21–23 (22; *n* = 10), total width 4–5 (4; *n* = 10). Ventral bar airplane shaped, five-pointed, total length 28–31 (30; *n* = 10), total width 22–27 (24; *n* = 10); Marginal hooks 7 pairs, dissimilar in size, each with delicate point, long shaft with expanded proximal subunit; filament loop partial, reaching close to level of expanding part of shaft. Hook lengths (*n* = 10): pair I 21–22 (21), pair II 19–24 (21), pair III 22–26 (25), pair IV 25–32 (28), pair V 21–23 (22), pair VI 20–24 (22), pair VII 22–28 (24). One pair of needles located near marginal hooks of pair V, length 11–12 (12; *n* = 10). Vagina sclerotized, elongated, usually twisted tube, with anchor shaped opening (opens dextrally), trace length 54–62 (56; *n* = 10). MCO comprising basally articulated copulatory tube and accessory piece, total length 34–38 (36; *n* = 10). Copulatory tube delicate, undulated in its medial part, distally narrowing to non-enveloped termination, tube-trace length 45–54 (49; *n* = 10); with thick-walled base, length 8–10 (8; *n* = 10), width 6–7 (6; *n* = 10). Accessory piece passing to colon-shaped process encircling medial part of copulatory tube, in distal portion and shield-like membranous broadening supporting copulatory tube.

Remarks

According to the morphology of the haptoral hard parts and reproductive organs, *D. omenti* n. sp. is most similar to *Dactylogyrus affinis* Bychowsky, 1933 (recorded from *Barbus lacerta* Heckel, 1843 [74], *Luciobarbus brachycephalus* (Kessler, 1872) [75], *L. capito* (Güldenstädt, 1773) [76] and *L. xanthopterus* Heckel, 1843 [77]), *Dactylogyrus deziensioides* Gussev, Jalali & Molnar, 1993 (from *L. kersin* Heckel, 1843 [78]), and *Dactylogyrus crivellius* (from *B. prespensis*) [48, 79]. However, *D. omenti* n. sp. differs from these species by the size of its haptoral hard parts, which are smaller (comparative morphometric data are provided in Table 4). In general, the configuration of hard haptoral elements and the shape of the ventral bars also resembles *Dactylogyrus* spp. from Moroccan *Luciobarbus* spp. described by el Gharbi et al. [80]. The MCO of *D. omenti* n. sp. most closely resembles the MCO of *D. deziensioides*, due to the presence of the colon-shaped process of the accessory piece encircling the copulatory tube. However, the copulatory tube of *D. deziensioides* is massive and short, in contrast with the delicate and long copulatory tube of *D. omenti* n. sp. In the original description of *D. affinis*, Bychowsky [81] pointed out the poor visibility of the end of copulatory tube, because of a saucer-shaped broadening of the accessory piece. This observation corresponds with the poor visibility of the medial part of the copulatory tube of *D. omenti* n. sp., on account of the shield-like broadening. Nevertheless, the colon-shaped process of the accessory piece is missing in the original drawing of *D. affinis.* The elongated twisted vagina of *D. affinis* markedly resembles the shape of the vagina of *D. omenti* n. sp. In regards to *D. crivellius*, *D. omenti* n. sp. differs in having a longer copulatory tube, larger colon-shaped part of the accesory piece and a thinner and longer vagina.

Discussion

With two species now known, the overall species richness of *Dactylogyrus* from *A. huegelii* is similar to that of other *Barbus* species from southern (France and Spain) and central Europe, for which 1–3 *Dactylogyrus* species per host species have been documented [25, 82]. The species richness of *Dactylogyrus* from *Barbus* species in the Balkan Peninsula ranges between 1 and 5 *Dactylogyrus* species per host species [e.g. 48]. While endemic and widely distributed *Barbus* species share several *Dactylogyrus* species (such as *D. dyki*, *D. petenyi*, *D. crivellius*, *D. carpathicus*, *D. malleus* and *D. balkanicus*), *D. omenti* n. sp. was recognized only from *A. huegelii* in this study, and therefore it is likely specific for this cyprinid species.

Dactylogyrus vastator, the parasite species with a large body size, has been widely reported from wild and farmed populations of *C. carpio* and *Carassius* spp., both of which belong to the subfamily Cyprininae (e.g. [52, 83–86]). In addition, the accidental infection of *D. vastator* was also

Table 4 Comparative metrical data (in μm) for hard parts of the haptor and reproductive organs of *D. omenti* n. sp. and morphologically similar *Dactylogyrus* spp.

Character		*D. omenti* n. sp.	*D. affinis*	*D. deziensioides*	*D. crivellius*
Body	length	230–522	600[a]	470[a]	–
	width	57–128	160[a]	120[a]	–
Anchors	inner length	37–41	46–65	47–49	58–61
	outer length	34–37	39–50	35–37	49–52
	inner root length	11–16	12–21	16–17	19–20
	outer root length	3–6	3–6	5–6	7–8
	point length	6–7	12–15	12–14	17–18
Ventral bar	length	28–31	50[a]	43–47	42[a]
	width	22–27	34[a]	30–32	26[a]
Dorsal bar	length	21–23	36–46	33[a]	42–43
	width	4–5	4–8	3–4	9[a]
Marginal hooks	length	19–32	21–33	25–28	31–34
Needle	length	11–12	–	–	–
MCO	length	34–38	37–47	46[a]	58–62
Vagina	length	54–62	40–50	–	–

[a]Maximum values of measured trait

Measurements of *D. affinis, D. deziensioides* and *D. crivellius* are obtained from [91]

found on some other cyprinid species (especially on *Barbus* [13], M. Benovics, unpublished data). Our study revealed a moderate prevalence of *D. vastator* on *A. huegelii*, which indicates that the infection of *D. vastator* on this endemic cyprinid species is not an accident. However, the low parasite infrapopulation size may indicate that this host is probably not suitable for maintaining parasite populations. *Cyprinus carpio* and *C. gibelio* may harbour up to nine different *Dactylogyrus* species [25, 87, 88]. The presence of only *D. vastator* on *A. huegelii* from this wide range of *Dactylogyrus* species could indicate: (i) the absence of other *Dactylogyrus* spp. on *C. carpio* and *Carassius* species potentially living in sympatry with *A. huegelii*; (ii) strict host specificity among other *Dactylogyrus* spp. of *C. carpio* and *C. gibelio* resulting from reciprocal co-adaptation; or (iii) different morphologies of gill filaments providing microhabitats suitable for some *Dactylogyrus* species (i.e. large species such as *D. vastator* or *D. extensus*), but unsuitable for others (i.e. small species such as *D. achmerowi* Gussev, 1955, *D. falciformis* Akhmerov, 1952 or *D. minutus* Kulwiec, 1927). To test these hypotheses, further investigation of parasite communities on *C. carpio* and *Carassius* spp. potentially living in sympatry with *A. huegelli* and analyses of the niche preferences of *Dactylogyrus* parasites (i.e. the preferred positions on fish gills) are necessary. *Dactylogyrus vastator* usually infects small fingerlings, where overpopulation may result in the mortality of the host. According to Uspenskaya [89], 40 specimens of *D. vastator* could possibly cause the death of a fish with a body length of 2 cm. This is not the case with *A. huegelii*, where very low abundance was found, i.e. only a single specimen of *D. vastator* per individual fish, suggesting that mortality of this host is unlikely. This low abundance is conflicting with optimal conditions for the development of this parasite species [90, 91], because high population growth and consequently a high intensity of infection on the part of *D. vastator* are expected in southern regions, which have high water temperatures in summer. Possible explanations could be that the mobility of *D. vastator* larvae is restricted by different suboptimal environmental factors, resulting from the habitat preference of *A. huegelii*; that is, finding new hosts in these conditions may be more difficult. Alternatively, this species could be competitively excluded by higher populations of the second host-specific species parasitizing *A. huegelii*, *Dactylogyrus omenti* n. sp. [79, 92], which, in our study, was the most abundant *Dactylogyrus* species on *A. huegelli*.

We hypothesized that *Dactylogyrus* species are a good indicator of evolutionary relationships between cyprinid host species. Despite the low abundance of *D. vastator* on *A. huegelii*, this record supports the phylogenetic relationships of *A. huegelii* to species of the Cyprininae originating from Asia and probably introduced into Europe, i.e. *C. carpio* and *Carassius* spp. This parasite species was also found in very low abundance (1 specimen per fish and a prevalence of 20%) on *Barbus plebejus* during our field study in Italy. *Aulopyge huegelii* possibly offers a similar type of substrate, which, in the case of *Dactylogyrus* spp., is gill filaments, and, therefore, common *Dactylogyrus* spp. parasitizing *C. carpio* and *Carassius* species [83, 85, 88, 93] can also develop and inhabit closely phylogenetically related species such as *A. huegelii* and some *Barbus* species. This may support the finding of Shamsi et al. [88] indicating that the transmission of *D. anchoratus* from common carp to *Barbus sharpeyi*, an important native fish species, takes place despite the high host specificity of many *Dactylogyrus* species. Šimková et al. [25] proposed that the phylogeny of *Dactylogyrus* reflects, at least partially, the phylogeny of their cyprinid host species (depending more or less on the level of host specificity of particular species). According to Kohlmann et al. [94], European and Asian cyprinids share a common ancestor from central Eurasia. While *C. carpio* is widely distributed in the Eurasian region, species of the *C. auratus* complex are native to eastern Asia and were only recently imported into Europe and other continents [26, 95]. There are no paleontological records of the *C. auratus* complex in Europe before the Pleistocene [95]. By computing pairwise genetic distances between *D. vastator* from different host species, we showed that *D. vastator* of *A. huegelii* collected in Bosnia and Herzegovina was genetically identical with *D. vastator* of *C. gibelio* from Croatia and *Barbus plebejus* collected in Italy. Moreover, this form of *D. vastator* appears to be evolutionarily closer to *D. vastator* collected from *C. carpio* than to *D. vastator* from *C. auratus* and *C. gibelio* from central Europe. However, as we have only limited data on the distribution of *D. vastator* in *C. carpio* or *Carassius* spp., and no data on the distribution and origin of these fish species in Mediterranean areas (the Apennine and Balkan Peninsulas), this may indicate two scenarios of historical dispersion of *D. vastator*: (i) *D. vastator* occurring in endemic Mediterranean fishes originated from the historical dispersion of *C. carpio* to the Mediterranean Peninsulas, where former population of *D. vastator* parasitizing non-native *C. carpio* switched to phylogenetically related Mediterranean cyprinid species and introduced *C. gibelio*, and then slightly genetically differentiated from the former population; (ii) Genetic differentiation took place among geographically isolated populations of *D. vastator* parasitizing *C. carpio* and the representatives of *C. auratus* complex, and the genetically differentiated form of *D. vastator* was, with their non-native hosts (probably with *C. gibelio*), introduced more recently to different Mediterranean Peninsulas and switched to phylogenetically related endemic Mediterranean cyprinids. Both scenarios may suggest the potential risk of *D. vastator* infection for endemic cyprinids. Data on the infection levels of *D. vastator* in non-native *C. carpio* and *C. gibelio* in Mediterranean areas may be helpful to clarify whether endemic cyprinids serve as real or accidental host species for this species. Unfortunately, such data are not at disposal in this study.

The phylogenetic position of *D. omenti* n. sp. was found to be nested within *Dactylogyrus* of *Barbus* and *Luciobarbus*. The morphological similarity between the copulatory organs and haptoral hard parts of *D. omenti* n. sp. and *D. affinis* and *D. deziensioides* indicates the potentially earlier diversion of the newly described species from species parasitizing *Barbus* and *Luciobarbus* species from Kazakhstan, Turkey and Middle East. This supports the close phylogenetic affinity of *A. huegelii* with ancestral *Barbus* lineages of Asia, from which *A. huegelii* and European *Barbus* lineages supposedly emerged [43]. Unfortunately, the lack of molecular data for *D. affinis* and *D. deziensioides* makes further examination of evolutionary connections currently impossible. With the shape of its haptoral hard elements, especially its typical cross-shaped ventral bar with five extremities, *D. omenti* n. sp. resembles *Dactylogyrus* of Greek and Moroccan *Luciobarbus* (see [80] for their morphology) and also *D. carpathicus* and *D. crivellius* from widely distributed *Barbus* species [48, 82]. It was suggested that the shape of the haptoral hard parts appears to be more suitable for resolving phylogenetic relationships between lineages of a given monogenean genus, while the shape of the reproductive organs is more suitable for identification at the species level because of its faster evolutionary change [23, 96–99]. This may indicate that *D. omenti* n. sp. is evolutionarily closer to the earlier mentioned species than to other *Dactylogyrus* of *Barbus*, possessing a different type of ventral bar. Nevertheless, our results showed that four *Dactylogyrus* spp. with a cross-shaped ventral bar with 5 extremities, i.e. *D. crivellius*, *D. carpathicus*, *Dactylogyrus* sp. 1 and *Dactylogyrus* sp. 2 (clade B in Fig. 1), formed a well supported (PP = 0.95, BS = 64) monophyletic group to the exclusion of *D. omenti* n. sp. The unexpected phylogenetic position of *D. omenti* n. sp. indicates that using only the shape of the haptor as a marker for solving phylogenetic relationships in monogenean species with rapid diversification is not advisable and that the shape of the reproductive organs should be taken into account. However, the phylogenetic relationships between other *Dactylogyrus* species included in our phylogenetic reconstruction follow haptor morphology, specifically the shape of the connective bars and hooks. This is true of the monophyletic group of *D. balkanicus* and *D. dyki* (group C), which possess a similar shape of hard parts of attachment organ (anchors, connective bars and marginal hooks) [48], though the two species vary in the dimensions of their haptoral hard parts [79]. Additionally, the copulatory organ of both species is similar. The fast development of variations in reproduction organs is considered as a mechanism for avoiding hybridization in the case of multiple congeneric monogenean species living in overlapping microhabitats [100]. This is also true for *Dactylogyrus* species parasitizing *Barbus*. Of a possible seven *Dactylogyrus* spp., *Barbus* and *Luciobarbus* species usually harbour only *Dactylogyrus* species with copulatory organs of a markedly different shape, representing different phylogenetic lineages ([80, 92, 101]. For instance, as is shown in the present study, *B. prespensis* hosts four species with differently shaped copulatory organs, *D. balkanicus*, *D. crivellius*, *D. dyki* and *D. prespensis*, representing three different phylogenetic lineages (see Table 2 and Fig. 1). Also the morphologically similar and phylogenetically close species, such as *D. dyki* and *D. balkanicus*, were not present on the same host species in one population.

Conclusions

Dactylogylrus omenti n. sp. was recognized as a potentially strict specialist of *A. huegelii*. Concluding from the expected high degree of host specificity of *Dactylogyrus* parasites and presence of *D. vastator*, a typical parasite of *C. carpio* and *Carassius* spp., on *A. huegelii*, or the phylogenetic position of *D. omenti* n. sp., the *A. huegelii* is a taxon closely related to European *Barbus* and *Luciobarbus* and to the Cyprininae of Asian origin. Regarding hard morphological characters, *D. omenti* n. sp. resembles species of *Dactylogyrus* parasitizing species of *Barbus* and *Luciobarbus* from the Middle East and Kazakhstan. Similarities in the shape of hard parts may suggest the origin of *D. omenti* n. sp. in this region and also an evolutionary proximity of endemic Cyprininae from the Middle East and Kazakhstan to *A. huegelii*. The genetic distances between *D. vastator* collected from different host species revealed that *D. vastator* in *A. huegelii* is identical with *D. vastator* of Balkan *C. gibelio* and closer to the central European *C. carpio* rather than to *C. auratus* complex. These results are indicating recent host switch of *D. vastator* between different hosts in Europe. The phylogenetic reconstruction of *Dactylogyrus* species parasitizing different endemic *Barbus* spp. and *Luciobarbus* spp. in the Balkan Peninsula and widely distributed European *Barbus* spp. revealed that, despite the generally accepted view that the morphology of the attachment organ is the best tool for resolving phylogenetic relations (based on morphological characters only) between *Dactylogyrus* species, the shape and size of the copulatory organs of rapidly evolving monogeneans have to be taken into consideration. But most importantly, only the combination of both morphological characters together with molecular data should be used for resolving the phylogeny and detection of potentially hidden diversity.

Abbreviations

BI: Bayesian inference analysis; BS: Bootstrap values resulting from maximum likelihood analysis; GAP: Mixture of glycerine and ammonium picrate; MC^3: Metropolis-coupled Markov chain Monte Carlo analysis; MCO: Male copulatory organ; ML: Maximum likelihood analysis; PP: Posterior probability resulting from Bayesian inference analysis

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Availability of data and materials

The data supporting the conclusions of this study are included in this article. The type-material of the new species described in this study was deposited in the Helminthological Collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic under the accession number IPCAS M-629. The newly generated sequences were submitted to the GenBank database (accession numbers are detailed in Table 2).

Authors' contributions

AŠ designed and supervised the study and organised the field trip and parasite collection. AŠ, MB and MLK processed fish and collected parasites during the field trip. MB and MLK performed microscopical observations and identified new species. MLK drew the hard parts and wrote the description of the new species. MB performed all laboratory procedures and phylogenetic analyses. AŠ performed statistical analyses. AŠ and MB wrote the draft of the paper and discussed the results. AŠ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval

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).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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PAPER II

Host-specific *Dactylogyrus* parasites revealing new insights on the historical biogeography of Northwest African and Iberian cyprinid fish

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Host-specific *Dactylogyrus* parasites revealing new insights on the historical biogeography of Northwest African and Iberian cyprinid fish

Andrea Šimková[1*], Michal Benovics[1], Imane Rahmouni[2] and Jasna Vukić[3]

Abstract

Background: Host specificity in parasites represents the extent to which a parasite's distribution is limited to certain host species. Considering host-specific parasites of primarily freshwater fish (such as gill monogeneans), their biogeographical distribution is essentially influenced by both evolutionary and ecological processes. Due to the limited capacity for historical dispersion in freshwater fish, their specific coevolving parasites may, through historical host-parasite associations, at least partially reveal the historical biogeographical routes (or historical contacts) of host species. We used *Dactylogyrus* spp., parasites specific to cyprinid fish, to infer potential historical contacts between Northwest African and European and Asian cyprinid faunas. Using phylogenetic reconstruction, we investigated the origin(s) of host-specific *Dactylogyrus* spp. parasitizing Northwest African and Iberian cyprinid species.

Results: In accordance with hypotheses on the historical biogeography of two cyprinid lineages in Northwest Africa, Barbini (*Luciobarbus*) and Torini (*Carasobarbus*), we demonstrated the multiple origins of Northwest African *Dactylogyrus*. *Dactylogyrus* spp. of *Carasobarbus* spp. originated from Asian cyprinids, while *Dactylogyrus* spp. of *Luciobarbus* spp. originated from European cyprinids. This indicates the historical Northern route of *Dactylogyrus* spp. dispersion to Northwest African *Luciobarbus* species rather than the Southern route, which is currently widely accepted for *Luciobarbus*. In addition, both Northwest African cyprinid lineages were also colonized by *Dactylogyrus marocanus* closely related to *Dactylogyrus* spp. parasitizing African *Labeo* spp., which suggests a single host switch from African Labeonini to Northwest African *Luciobarbus*. We also demonstrated the multiple origins of *Dactylogyrus* spp. parasitizing Iberian *Luciobarbus* species. One Iberian *Dactylogyrus* group was phylogenetically closely related to *Dactylogyrus* of Moroccan *Carasobarbus*, while the second was related to *Dactylogyrus* of Moroccan *Luciobarbus*.

Conclusions: Our study confirms the different origins of two Northwest African cyprinid lineages. It suggests several independent historical contacts between European Iberian *Luciobarbus* and two lineages of Northwest African cyprinids, these contacts associated with host switches of *Dactylogyrus* parasites.

Keywords: Cyprinids, *Dactylogyrus*, Historical biogeography, Host specificity, Iberia, Northwest Africa

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* Correspondence: simkova@sci.muni.cz
[1]Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic
Full list of author information is available at the end of the article

Background

Primary freshwater fish are supposed to be intolerant to salinity, and thus their dispersal is restricted to freshwater routes only. Because of such limited dispersion mechanisms, relationships between fish lineages may reflect relationships between different areas; therefore, freshwater fish are suitable for studies of historical biogeography [1]. Over evolutionary time, the diversity of parasite communities of such freshwater fish is shaped by coevolutionary and historical biogeographical processes (e.g. [2, 3]). However, over ecological time, parasite biogeography is also influenced by the temporal and spatial variability in ecological factors [4, 5].

Concerning freshwater fish, the biogeography of their helminth parasites was shown to reflect historical processes related to the current distribution of their hosts. For example, helminth diversity in Mexican freshwater fishes is determined by the historical and contemporary biogeography of their hosts [6]. The distribution of the metazoan parasites of the sturgeon fish (Acipenseridae) was shown to be in accord with the historical biogeographical routes of these fishes [7].

The host specificity of fish parasites (i.e. the extent to which a parasite's distribution is limited to certain host species) seems to be their most important characteristic, with the potential to reflect historical host-parasite associations and to indicate the historical biogeographical routes of hosts. McDowell [8] showed that parasites not coevolving with their galaxioid fish hosts (i.e. Galaxiidae and Retropinnidae) do not support a vicariance biogeography for galaxioid fish. However, if the host specificity of a parasite group is high, then the phylogenetic and biogeographical relationships between hosts and parasites may be mutually illuminating [1].

Gill monogeneans of the highly diversified genus *Dactylogyrus* Diesing, 1850 are species-specific to their cyprinid host species (with some rare exceptions). According to Šimková et al. [9], *Dactylogyrus* species often exhibit strict host specificity (i.e. they are specific to a single cyprinid species), congeneric host specificity (i.e. they are specific to congeneric cyprinid species), or phylogenetic host specificity (i.e. they are specific to phylogenetically closely related cyprinid species). The distribution of *Dactylogyrus* species on their cyprinid hosts reflects the evolutionary history of these fishes [10]. The evolution of *Dactylogyrus* lineages is associated with different cyprinid lineages, and the presence of the same *Dactylogyrus* species on the representatives of different cyprinid lineages (i.e. in cyprinid species with high divergence but living in the same biogeographical area) is only accidental [9, 10]. Little is known about *Dactylogyrus* of cyprinid species living in the Mediterranean region. However, some studies are suggesting that due to high host specificity, the endemism of *Dactylogyrus* parasites follows the endemism of their cyprinid host species. Such endemic *Dactylogyrus* were documented for *Luciobarbus* Heckel, 1843 from the Iberian Peninsula [11], for *Luciobarbus* from Northwest Africa [12], and for cyprinids living in Lake Mikri Prespa (northern Greece) [13, 14].

Cyprinids are primarily freshwater fish with their native distribution in Europe, Asia, Africa and North America. The different cyprinid lineages exhibit different biogeographical distributions across continents [15]. One of the lineages, the subfamily Cyprininae, was recently revised by Yang et al. [16] to include 11 tribes. Most representatives of this subfamily inhabit waters of southern Eurasia and Africa. Of the four evolutionary lineages (i.e. tribes) of Cyprininae present in Africa, two have been recognized in Northwestern Africa. The first lineage includes hexaploid genera of large-sized barbels (*Carasobarbus* Karaman, 1971, *Pterocapoeta* Günther, 1902 and *Labeobarbus* Rüppel, 1835) belonging to the tribe Torini (this tribe includes large-sized barbels from Asia and Africa). The second lineage is represented by tetraploid *Luciobarbus* belonging to the tribe Barbini (this tribe includes the taxa distributed in Eurasia and Northwest Africa). Different origins and different dispersal events from Eurasia to Africa were proposed for these lineages. The two genera which are widespread in Northwest Africa, *Carasobarbus* and *Luciobarbus*, have disjunct distributions. *Carasobarbus* is distributed in Northwest Africa and the Middle East, while *Luciobarbus* is distributed in West Asia, Northwest Africa, Greece and the Iberian Peninsula. The large-sized African hexaploids are not monophyletic like the Moroccan *Carasobarbus* cluster with Middle East *Carasobarbus*, suggesting that the diversification of African hexaploids preceded the separation between the Middle East and Northwest African hexaploids [16, 17]. Tsigenopoulos et al. [17] suggested that the large hexaploids invaded Africa through the land bridge between Africa and Asia (via the Arabian tectonic Plate) formed in the Middle Miocene (about 13 MYA). Using molecular calibration, they calculated that the splitting of the African hexaploids from their Asian ancestors and subsequently the beginning of the diversification of the African hexaploid lineage occurred in the Late Miocene. The genus *Luciobarbus* is paraphyletic, as the clade also includes the genus *Capoeta* Güldenstädt, 1773. Concerning *Luciobarbus* species in Northwest Africa, they do not form a monophyletic group either, as two Northwest African species cluster with Iberian species [16, 18–20]. Concerning *Luciobarbus* in the Iberian Peninsula, three main hypotheses were proposed for their origin; some of them have direct implications for the origin of this genus in Northwest Africa. First, Banarescu [21] and

Almaça [22] proposed that the Iberian Peninsula was colonized from the North before the formation of the Pyrenees. Based on this hypothesis, barbels from the Iberian Peninsula are evolutionarily closer to European and African barbels than to Asian species. Secondly, Doadrio [23] proposed that *Luciobarbus* colonized Iberia from Africa via southern Spain at the Miocene-Pliocene boundary (about 5 MYA) after the Messinian salinity crisis of the Mediterranean Sea. Following this hypothesis, barbels from the Iberian Peninsula are phylogenetically closer to Asian and North African barbels than to those of central Europe. Thirdly, Bianco [24] proposed that the distribution of *Luciobarbus* be explained by the freshwater phase (the so-called Lago Mare phase) of the Mediterranean Sea, which supposedly followed the Messinian salinity crisis. Following this hypothesis, Iberian barbels are more related to those of the Balkans than to central European species. However, this third hypothesis has been rejected by many authors by both geological data and the estimation of the time of diversification of freshwater fish species [25] according to the finding of fossils preceding the given geological period. Tsigenopoulos et al. [26] and Yang et al. [16] showed that most *Luciobarbus* species from Northwest Africa are more closely related to *Luciobarbus* from the Middle East than to *Luciobarbus* from the Iberian Peninsula.

The aim of this study was to reconstruct the phylogeny of gill parasites of the genus *Dactylogyrus*, monogeneans specific to cyprinid fish species, to (i) investigate the phylogenetic position of African *Dactylogyrus* parasites in relation to European and Asian *Dactylogyrus* lineages with a special focus on the origin(s) of *Dactylogyrus* parasitizing Northwest African and Iberian cyprinid fish species, and (ii) infer potential scenarios of the *Dactylogyrus* colonization of Northwest African and Iberian cyprinids in relation to their historical biogeography.

Methods

Dactylogyrus species

For this study, *Dactylogyrus* species were sampled from cyprinid species in Morocco and the Iberian Peninsula. Other *Dactylogyrus* spp. collected from cyprinid species sampled in Europe (the Balkan Peninsula, including Greece and Bosnia and Herzegovina, and central Europe, represented by the Czech Republic) and Africa (Senegal) were included in this study. These *Dactylogyrus* spp. were selected to recover representatives parasitizing different cyprinid lineages and also to include species potentially phylogenetically related to the *Dactylogyrus* spp. collected in Northwest Africa and the Iberian Peninsula. In addition, *Dactylogyrus* species from Asian cyprinid species, for which molecular data (i.e. the sequences of 28S DNA) were available in GenBank, were included in the analyses. The list of studied *Dactylogyrus* species, their host species, locality of collection, and accession numbers are presented in Table 1.

In the field, *Dactylogyrus* species were removed from fish gills during fish dissection (following Ergens & Lom [27]), placed on slides, covered with a coverslip, and fixed in a mixture of glycerine and ammonium picrate (GAP). The identification was performed on the basis of the size and shape of the sclerotized parts of the attachment organ, the haptor, and the sclerotized parts of the reproductive organs, following the original descriptions [11, 12, 28–32]. Morphological examination was performed using an Olympus BX51 light microscope equipped with phase contrast and differential interference contrast. Some specimens of each *Dactylogyrus* species were bisected; one-half of the body (usually the anterior one with reproductive organs) was mounted on a slide for species identification, and the other was individually preserved in 96% ethanol for DNA extraction.

DNA extraction, amplification and sequencing

Dactylogyrus species collected from cyprinids in Africa and Europe were sequenced to obtain partial sequences of 28S rDNA and partial sequences of 18S rDNA and the ITS1 region. *Dactylogyrus* specimens were individually removed from ethanol and dried by using a vacuum centrifuge. Genomic DNA extraction was performed following a standard protocol (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Partial 28S rDNA was amplified using the forward primer C1 (5′-ACC CGC TGA ATT TAA GCA-3′) and the reverse primer D2 (5′-TGG TCC GTG TTT CAA GAC-3′) [33]. PCR followed the protocol included in Šimková et al. [34]. Partial 18S rDNA and the entire ITS1 region were amplified in one round using the primers S1 (5′-ATT CCG ATA ACG AAC GAG ACT-3′) and IR8 (5′-GCT AGC TGC GTT CTT CAT CGA-3′) [35] that anneal to 18S and 5.8S rDNA, respectively. Each amplification reaction for partial 18S rDNA and the ITS1 region was performed in a final volume of 15 μl, containing 1.5 U of *Taq* polymerase, 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 2.5 μl of DNA (20 ng/μl). PCR was carried out using the following steps: 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 53 °C and 1 min 30 s at 72 °C, and 10 min of final elongation at 72 °C. The PCR products were checked on 1.5% agarose gel, purified using ExoSAP-IT kit (Ecoli, SK) following a standard protocol and directly sequenced using the PCR primers and BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was carried out using an ABI 3130 Genetic Analyser (Applied Biosystems). Sequences were analysed

Table 1 List of *Dactylogyrus* species, their cyprinid host species, cyprinid phylogeny, country of collection and GenBank accession numbers for sequences used in the phylogenetic analyses

Dactylogyrus species	Cyprinid host species	Cyprinid subfamily	Cyprinid tribe	Country of collection	GenBank ID (28S rDNA)	GenBank ID (18S rDNA with ITS1)
D. bicornis Malewitzkaja, 1941[a]	*Rhodeus meridionalis* Karaman, 1924	Acheilognathinae	–	Greece	KY629345	–
D. labei Musselius & Gussev, 1976	*Catla catla* (Hamilton, 1822)	Cyprininae	Labeonini	India	JX566720	–
D. quanfami Ha Ky, 1971	*Cirrhinus molitorella* (Valenciennes, 1844)	Cyprininae	Labeonini	China	EF100536	–
D. lenkoranoides El Gharbi, Renaud & Lambert, 1992	*Luciobarbus guiraonis* (Steindachner, 1866)	Cyprininae	Barbini	Spain	KY629346	–
D. bocageii Alvarez Pellitero, Simón Vicente & González Lanza, 1981	*Luciobarbus bocagei* (Steindachner, 1864)	Cyprininae	Barbini	Portugal	KY629347	–
D. balistae Simón Vicente, 1981	*Luciobarbus bocagei*	Cyprininae	Barbini	Portugal	–	KY629344
D. mascomai El Gharbi, Renaud & Lambert, 1992	*Luciobarbus guiraonis*	Cyprininae	Barbini	Spain	KY629348	–
D. linstowoides El Gharbi, Renaud & Lambert, 1992	*Luciobarbus guiraonis*	Cyprininae	Barbini	Spain	KY629349	KY629329
D. legionensis González Lanza & Alvarez Pellitero, 1982	*Luciobarbus guiraonis*	Cyprininae	Barbini	Spain	KY629350	KY629330
D. andalousiensis El Gharbi, Renaud & Lambert, 1992	*Luciobarbus sclateri* Günther, 1868	Cyprininae	Barbini	Portugal	KY629351	KY629331
D. zatensis El Gharbi, Birgi & Lambert, 1994	*Carasobarbus fritschii* Günther, 1874	Cyprininae	Torini	Morocco	KY629352	KY629335
D. volutus El Gharbi, Birgi & Lambert, 1994	*Carasobarbus fritschii*	Cyprininae	Torini	Morocco	KY629353	KY629334
D. kulindrii El Gharbi, Birgi & Lambert, 1994	*Carasobarbus fritschii*	Cyprininae	Torini	Morocco	KY629354	KY629336
D. marocanus El Gharbi, Birgi & Lambert, 1994[b]	*Carasobarbus fritschii,*	Cyprininae	Torini	Morocco	KY629355	KY629333
D. scorpius Rahmouni, Řehulková & Šimková, 2017	*Luciobarbus rifensis* Doadrio, Casal-Lopéz & Yahyaoui, 2015	Cyprininae	Barbini	Morocco	KY553860	KY578023
D. benhoussai Rahmouni, Řehulková & Šimková, 2017	*Luciobarbus moulouyensis* (Pellegrin, 1924)	Cyprininae	Barbini	Morocco	KY553862	KY578025
D. varius Rahmouni, Řehulková & Šimková, 2017	*Luciobarbus maghrebensis* Doadrio, Perea & Yahyaoui, 2015	Cyprininae	Barbini	Morocco	KZ553863	KY578026
D. falsiphallus Rahmouni, Řehulková & Šimková, 2017	*Luciobarbus maghrebensis*	Cyprininae	Barbini	Morocco	KZ553861	KY578024
D. atlasensis El Gharbi, Birgi & Lambert, 1994	*Luciobarbus pallaryi* (Pellegrin, 1919)	Cyprininae	Barbini	Morocco	KY629356	KY629337
D. fimbriphallus El Gharbi, Birgi & Lambert, 1994	*Luciobarbus massaensis* (Pellegrin, 1922)	Cyprininae	Barbini	Morocco	KY629357	KY629332
Dactylogyrus sp. 1	*Enteromius niokoloensis* (Daget, 1959)	Cyprininae	Smiliogastrini	Senegal	KY629358	–
D. aspili Birgi & Lambert, 1987	*Enteromius macrops* (Boulenger, 1911)	Cyprininae	Smiliogastrini	Senegal	KY629359	–
D. leonis Musilová, Řehulková & Gelnar, 2009	*Labeo coubie* Rüppell, 1832	Cyprininae	Labeonini	Senegal	KY629360	–
D. oligospirophallus Paperna, 1973	*Labeo coubie*	Cyprininae	Labeonini	Senegal	KY629361	–
D. brevicirrus Paperna, 1973	*Labeo parvus* Boulenger, 1902	Cyprininae	Labeonini	Senegal	KY629362	–
D. senegalensis Paperna, 1969	*Labeo senegalensis* Valenciennes, 1842	Cyprininae	Labeonini	Senegal	KY629363	–
D. titus Guégan, Lambert & Euzet, 1988	*Labeo senegalensis*	Cyprininae	Labeonini	Senegal	KY629364	–
D. falcilocus Guegan, Lambert & Euzet, 1988	*Labeo coubie*	Cyprininae	Labeonini	Senegal	KY629365	–
D. vastator Nybelin, 1924	*Carassius gibelio* (Bloch, 1782)	Cyprininae	Cyprinini	Czech Republic	KY629366	KY201103

Table 1 List of *Dactylogyrus* species, their cyprinid host species, cyprinid phylogeny, country of collection and GenBank accession numbers for sequences used in the phylogenetic analyses *(Continued)*

Dactylogyrus species	Cyprinid host species	Cyprinid subfamily	Cyprinid tribe	Country of collection	GenBank ID (28S rDNA)	GenBank ID (18S rDNA with ITS1)
D. extensus Mueller & Van Cleave, 1932	*Cyprinus carpio* Linnaeus, 1758	Cyprininae	Cyprinini	Czech Republic	AY553629	–
D. inexpectatus Isjumova in Gussev, 1955	*Carassius gibelio*	Cyprininae	Cyprinini	Czech Republic	AJ969945	–
D. anchoratus (Dujardin, 1845)	*Carassius gibelio*	Cyprininae	Cyprinini	Czech Republic	KY201116	KY201102
Dactylogyrus sp. AC2012	*Cyprinus carpio*	Cyprininae	Cyprinini	India	JQ926198	–
D. dyki Ergens & Lucky, 1959	*Barbus barbus* (Linnaeus, 1758)	Cyprininae	Barbini	Czech Republic	KY629367	KY629338
D. crivellius Dupont & Lambert, 1986	*Barbus peloponesius* Valenciennes, 1842	Cyprininae	Barbini	Greece	KY629368	KY629339
D. carpathicus Zachvatkin, 1951	*Barbus barbus*	Cyprininae	Barbini	Czech Republic	KY201111	KY201098
Dactylogyrus sp. 2	*Luciobarbus albanicus* (Steindachner, 1870)	Cyprininae	Barbini	Greece	KY201114	KY201100
Dactylogyrus sp. 3	*Luciobarbus graecus* (Steindachner, 1895)	Cyprininae	Barbini	Greece	KY201115	KY201101
D. prespensis Dupont & Lambert, 1986	*Barbus prespensis* Karaman, 1924	Cyprininae	Barbini	Greece	KY201110	KY201096
D. petenyi Kastak, 1957	*Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002	Cyprininae	Barbini	Greece	-	KY201097
D. malleus Linstow, 1877	*Barbus barbus*	Cyprininae	Barbini	Czech Republic	KY201112	KY201099
D. vistulae Prost, 1957	*Squalius prespensis* (Fowler, 1977)	Leuciscinae	–	Albania	KY629369	KY629640
D. fallax Wagener, 1857	*Vimba vimba* (Linnaeus, 1758)	Leuciscinae	–	Czech Republic	KY629370	KY629341
D. cornu Linstow, 1878	*Vimba vimba*	Leuciscinae	–	Czech Republic	KY629371	KY629342
D. borealis Nybelin, 1937	*Phoxinus* sp.	Leuciscinae	–	Bosnia and Herzegovina	KY629372	KY629343
D. nanus Dogiel & Bychowsky, 1934	*Rutilus rutilus* (Linnaeus, 1758)	Leuciscinae	–	Czech Republic	AJ969942	AJ564145
D. sphyrna Linstow, 1878	*Rutilus rutilus*	Leuciscinae	–	Czech Republic	AJ969943	AJ564154
D. suecicus Nybelin, 1937	*Rutilus rutilus*	Leuciscinae	–	Czech Republic	KY629373	–
D. crucifer Wagener, 1857	*Rutilus rutilus*	Leuciscinae	–	Czech Republic	KY629374	AJ564120
D. wunderi Bychowsky, 1931	*Abramis brama* (Linnaeus, 1758)	Leuciscinae	–	Czech Republic	KY629375	AJ564164
D. cryptomeres Bychowsky, 1943	*Gobio gobio* (Linnaeus, 1758)	Gobioninae	–	Czech Republic	AJ969947	–
D. lamellatus Achmerow, 1952	*Ctenopharyngodon idella* (Valenciennes, 1844)	Xenocyprinae	–	China	AY307019	–
D. hypophthalmichthys Akhmerov, 1952	*Hypophthalmichthys molitrix* (Valenciennes, 1844)	Xenocyprinae	–	China	EF100532	–
Dactylogyrus sp. (YY)	*Hypophthalmichthys nobilis* (Richardson, 1845)	Xenocyprinae	–	China	EF100538	–
D. parabramis Akhmerov, 1952	*Megalobrama terminalis* (Richardson, 1846)	Xenocyprinae	–	China	EF100534	–
D. petruschewskyi Gussev, 1955	*Megalobrama amblycephala* Yih, 1955	Xenocyprinae	–	China	AY548927	–
D. pekinensis Gussev, 1955	*Megalobrama amblycephala*	Xenocyprinae	–	China	EF100535	–

[a] Morphologically identical *D. bicornis* was also found on *Rhodeus amarus* (Bloch, 1782) from the Czech Republic; the sequence data are not available

[b] Morphologically and genetically identical *D. marocanus* was also collected from *Pterocapoeta maroccana*, *Luciobarbus ksibii*, *Luciobarbus zayanensis*

using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA), and new sequences were deposited in GenBank (see Table 1 for accession numbers). The sequences of other *Dactylogyrus* species parasitizing European and Asian cyprinid species were retrieved in GenBank (Table 1) and were used for phylogenetic analyses.

Phylogenetic analyses

The first alignment included the partial 28S rDNA sequences of 55 *Dactylogyrus* species. Among them, 36 were newly sequenced for this study. The sequences of the other 19 *Dactylogyrus* species as well as the sequences of three species of the Dactylogyridae

(*Euryhaliotrematoides pirulum* Plaisance & Kritsky, 2004, *Euryhaliotrematoides triangulovagina* Yamaguti, 1968 and *Aliatrema cribbi* Plaisance & Kritsky, 2004 with accession numbers AY820618, AY820619 and AY820612, respectively), used as the outgroup in the phylogenetic analyses, were retrieved from GenBank. The second alignment included the partial 18S rDNA sequences and the ITS1 region of 26 *Dactylogyrus* species belonging to *Dactylogyrus* lineage III. *D. vistulae* Prost, 1957 and *D. sphyrna* Linstow, 1978 were used as the outgroup in the phylogenetic analyses based on the 18S rDNA and ITS1 sequences.

All sequences of a given dataset were aligned using ClustalW multiple alignments [36] in Bioedit v. 7.2.5 [37]. The phylogenetic analyses were performed using unambiguous alignments. Gaps and ambiguously aligned regions were removed from alignments using GBlocks v. 0.91 [38]. The best-fit DNA evolution model was determined using the Akaike's information criterion (AIC) in JmodelTest 2.1.10 [39, 40]. Phylogenetic trees were inferred using minimum evolution (ME) analysis using PAUP* 4b10 [41], maximum likelihood (ML) analysis using PhyML 3.0 [42], and Bayesian inference (BI) analysis using MrBayes 3.2 [43]. Supports for internal nodes were computed from a bootstrap re-sampling procedure [44] with 1000 pseudoreplicates for ME, and 500 pseudoreplicates for ML using the TBR algorithm. A search for the best ML tree was performed using the TBR branch-swapping algorithm. Bayesian inference (BI) analyses were performed using four Monte Carlo Markov chains running on 1000,000 generations for each data set, with trees being sampled every 100 generations. The "burn-in" asymptote was estimated by plotting the number of generations against the log likelihood scores for the saved trees, and all the trees (25%) before stationarity were discarded as "burn-in". The posterior probabilities of the phylogeny and its branches were determined for all trees left in the plateau phase with the best ML scores.

The mapping of characters was performed in Mesquite 3.2 [45]. Prior to the mapping of characters, a new alignment was prepared using partial 28S rDNA sequence data from 55 *Dactylogyrus* species. Phylogenetic reconstruction using BI analysis was performed as described above. *Dactylogyrus bicornis* Malewitzkaja, 1941 was used for rooting the phylogenetic tree following the output of phylogenetic analyses using the external outgroup. The first character mapped onto the phylogenetic reconstruction represents fish lineages, i.e. different fish families as applied in Yang et al. [16] (Acheilognathinae, Xenocyprinae, Gobioninae, Leuciscinae and Cyprininae as different character states). The second character represents fish lineages including the branching within Cyprininae, the target group of our study (Cyprinini, Labeonini, Torini, Smiliogastrini, Barbini including the genus *Barbus* Cuvier & Cloquet, 1816, and Barbini including the genus *Luciobarbus* were used as the character states). The revised classification of the subfamily Cyprininae by Yang et al. [16] was adopted for this mapping. The last character represents the distribution of host species with the following character states applied: southern Asia including Southeast Asia, a large part of Eurasia, Europe with only West Asia, the Iberian Peninsula, the Balkan Peninsula, Northwest Africa and West Africa. The distribution of cyprinid species follows Froese & Pauly [46].

Results

An unambiguous alignment including the 55 *Dactylogyrus* species analysed and three outgroup species spanned 544 positions. The TVM + I + G model was selected as the best-fit evolutionary model. The ME, ML and BI analyses provided phylogenetic trees with similar topologies. The BI tree is presented in Fig. 1, including bootstrap values resulting from ME and ML analyses and posterior probabilities resulting from BI analysis. The phylogenetic reconstructions revealed four *Dactylogyrus* lineages with *D. bicornis* in the basal position (Fig. 1). *Dactylogyrus* lineage I included two *Dactylogyrus* species parasitizing Asian Labeonini in the basal position, and the monophyletic group including 3 *Dactylogyrus* parasitizing Iberian *Luciobarbus* (the tribe Barbini within Cyprininae) and *Dactylogyrus* parasitizing Northwest African *Carasobarbus fritschii* (Günther, 1874) (the tribe Torini within Cyprininae) (Table 1, Fig. 1). The other three *Dactylogyrus* lineages (II, III and IV) formed a clade well supported by BI analysis but weakly supported by ME and unsupported by ML. *Dactylogyrus* lineage II included two groups of African *Dactylogyrus*. The first group included *Dactylogyrus* parasitizing small *Enteromius* Cope, 1867 species (Smiliogastrini) collected in West Africa (the basal position of this group was weakly supported by PP resulting from BI analysis and BP resulting from ML analysis and unsupported by BP resulting from ME analysis). The second group included *Dactylogyrus* species parasitizing West African *Labeo* Cuvier, 1816 (Labeonini) with the nested position of a single *Dactylogyrus* species (*D. marocanus* El Gharbi, Birgi & Lambert, 1994) from Northwest African cyprinins of the tribes Barbini and Torini. *Dactylogyrus* lineage II also included *Dactylogyrus* species parasitizing *Cyprinus carpio* Linnaeus, 1758 and the complex of *Carassius auratus* (Linnaeus, 1758), two species of Asian origin recently widely distributed in Europe. *Dactylogyrus* lineage III included the species collected from Europe and parasitizing Leuciscinae species, *Barbus* species (Barbini, Cyprininae) with a European distribution, and the Northwest African *Luciobarus* (Table 1). Phylogenetic relationships within *Dactylogyrus* lineage III

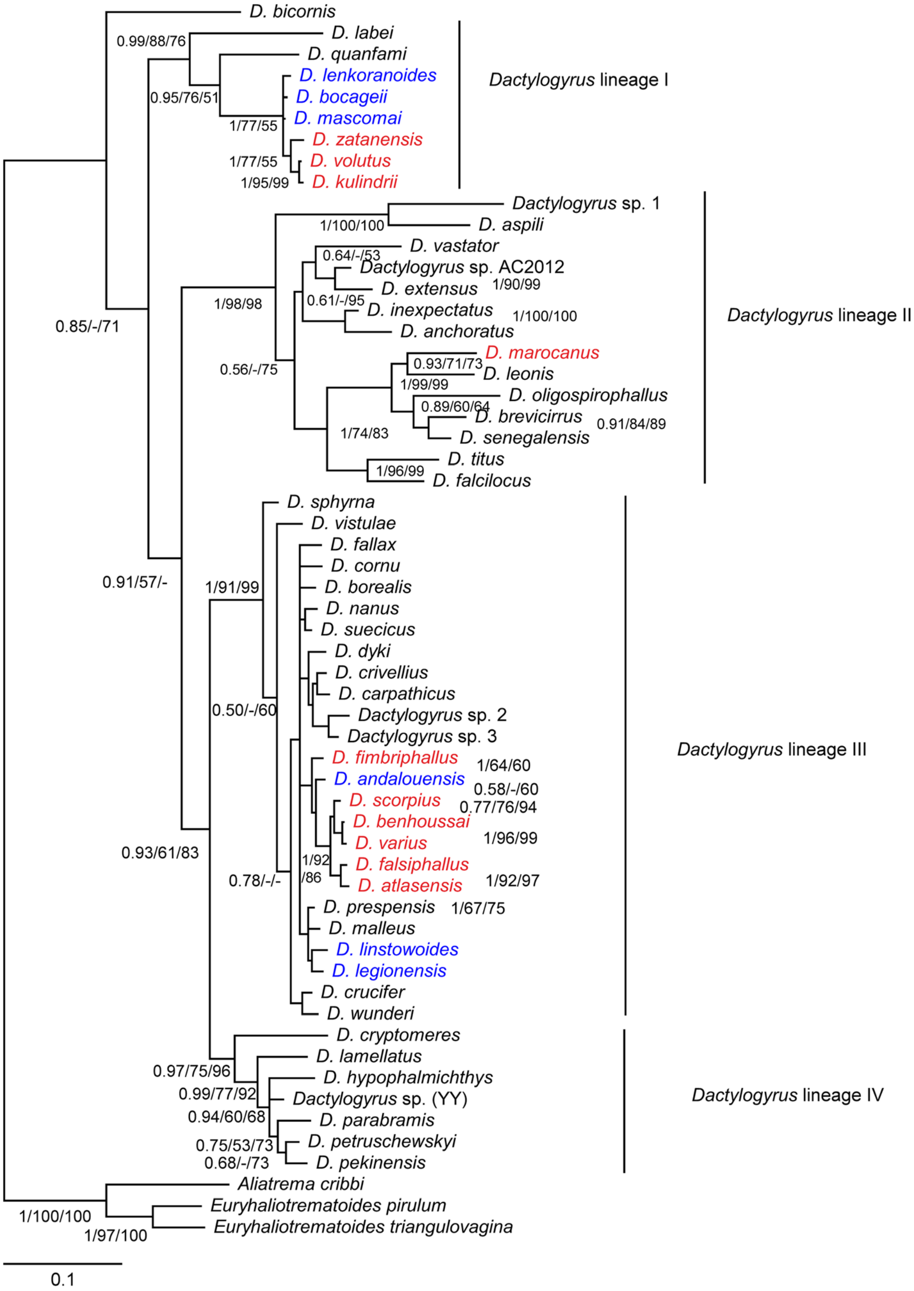

Fig. 1 Bayesian inference phylogenetic tree of *Dactylogyrus* species parasitizing different cyprinid lineages based on sequences of partial 28S rDNA. *Dactylogyrus* spp. from Northwest African cyprinids are shown in red. *Dactylogyrus* spp. from Iberian cyprinids are shown in blue. Values along branches indicate posterior probabilities and bootstrap values resulting from the following analyses: BI/ME/ML. *Abbreviations*: BI, Bayesian inference; ME, minimum evolution; ML, maximum likelihood. *Aliatrema cribbi*, *Euryhaliotrematodes pirulum* and *E. triangulovagina* were used as the outgroup

were either weakly resolved or unresolved by phylogenetic analyses. However, the monophyletic group including *Dactylogyrus* parasitizing Northwest African *Luciobarbus* species and *D. andalousiensis* El Gharbi, Renaud & Lambert, 1992 parasitizing Iberian *Luciobarbus sclateri* Günther, 1868 was either well or moderately supported by our phylogenetic analyses. *Dactylogyrus* lineage IV included *D. cryptomeres* Bychowsky, 1943 parasitizing cyprinids of Gobioninae in the basal position and the well-supported monophyletic group of *Dactylogyrus* parasitizing Asian Xenocyprinae (Table 1, Fig. 1).

Because of the impossibility of reconstructing a reliable alignment when including *Dactylogyrus* species of highly diversified cyprinid lineages (i.e. because of the presence of many hypervariable regions and indels), we used only the representatives of *Dactylogyrus* lineage III in subsequent phylogenetic analyses to resolve the phylogenetic relationships within this lineage. An unambiguous alignment including *Dactylogyrus* species of lineage III spanned 1072 positions. The GTR + I + G model was selected as the best-fit evolutionary model. The ME, ML and BI analyses provided phylogenetic trees with similar topologies. The BI tree is presented in Fig. 2, including bootstrap values resulting from ME and ML analyses and posterior probabilities resulting from BI analysis. The basal position of *D. andalousiensis* in relation to the monophyletic group of *Dactylogyrus* species parasitizing Moroccan *Luciobarbus* was well supported by PP resulting from BI analysis and BP resulting from ME analysis, and moderately supported by BP resulted from ML analysis. Three *Dactylogyrus* species parasitizing Iberian *Luciobarbus* species formed a monophyletic group with two *Dactylogyrus* parasitizing Balkan *Barbus* species and one *Dactylogyrus* parasitizing *Barbus* species with a wide European distribution. This cluster was well supported by all phylogenetic analyses.

The mapping of characters was performed in the phylogenetic reconstruction (BI tree) of 55 *Dactylogyrus* species. An unambiguous alignment spanned 568 positions. The GTR + I + G model was selected as the best evolutionary model. The mapping of the character of cyprinid lineages (i.e. cyprinid subfamilies) onto the phylogenetic reconstruction (Fig. 3) showed that Acheilognathinae is the most plesiomorphic host group for *Dactylogyrus*. *Dactylogyrus* of the Cyprininae are included in three lineages. The Gobioninae, Xenocyprinae and Leuciscinae were likely colonized by *Dactylogyrus* from the Cyprininae. However, some Cyprininae were secondarily colonized by *Dactylogyrus* from the Leuciscinae. The mapping of the cyprinid distribution onto the phylogenetic reconstruction (Fig. 4) showed the Asian origin of *Dactylogyrus*. This mapping revealed (i) the multiple origins of Northwest African *Dactylogyrus*, and (ii) the phylogenetic relatedness between *Dactylogyrus* parasitizing the Cyprininae of Labeonini, Cyprinini, Torini and some of Barbini across different continents. Northwest

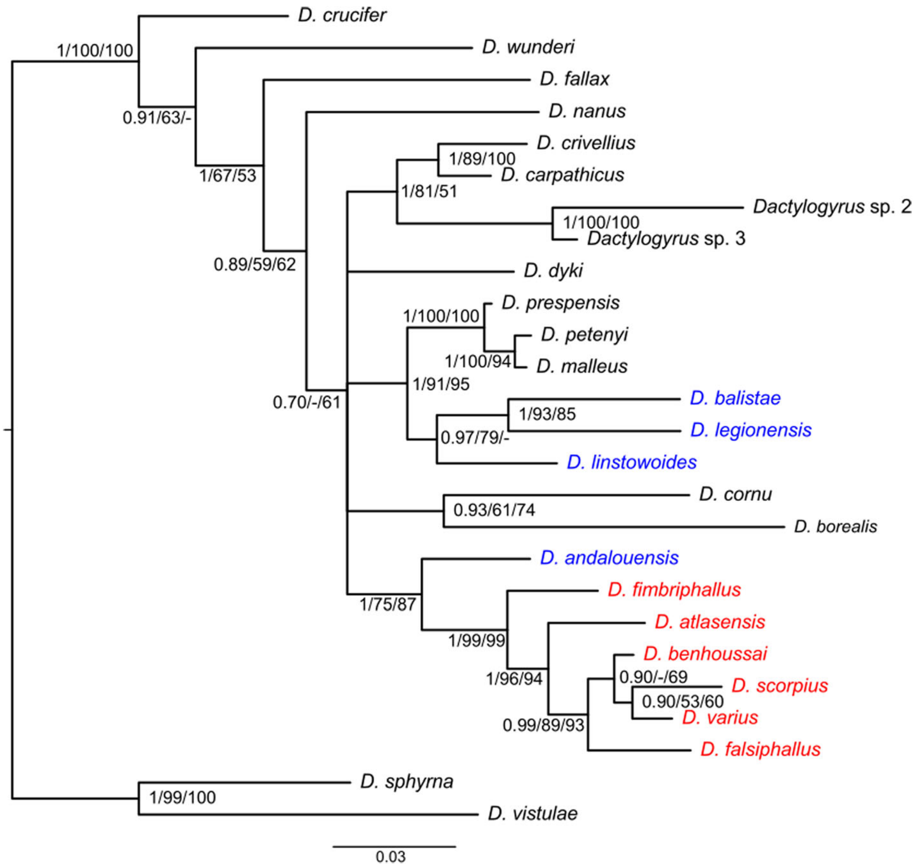

Fig. 2 Phylogenetic tree of *Dactylogyrus* species belonging to *Dactylogyrus* lineage III constructed by Bayesian inference analysis. The tree is based on sequences of partial 18S rDNA and ITS1. *Dactylogyrus* spp. from Northwest African cyprinids are shown in red. *Dactylogyrus* spp. from Iberian cyprinids are shown in blue. Values along branches indicate posterior probabilities and bootstrap values resulting from the following analyses: BI/ME/ML. *Abbreviations*: BI, Bayesian inference; ME, minimum evolution; ML, maximum likelihood

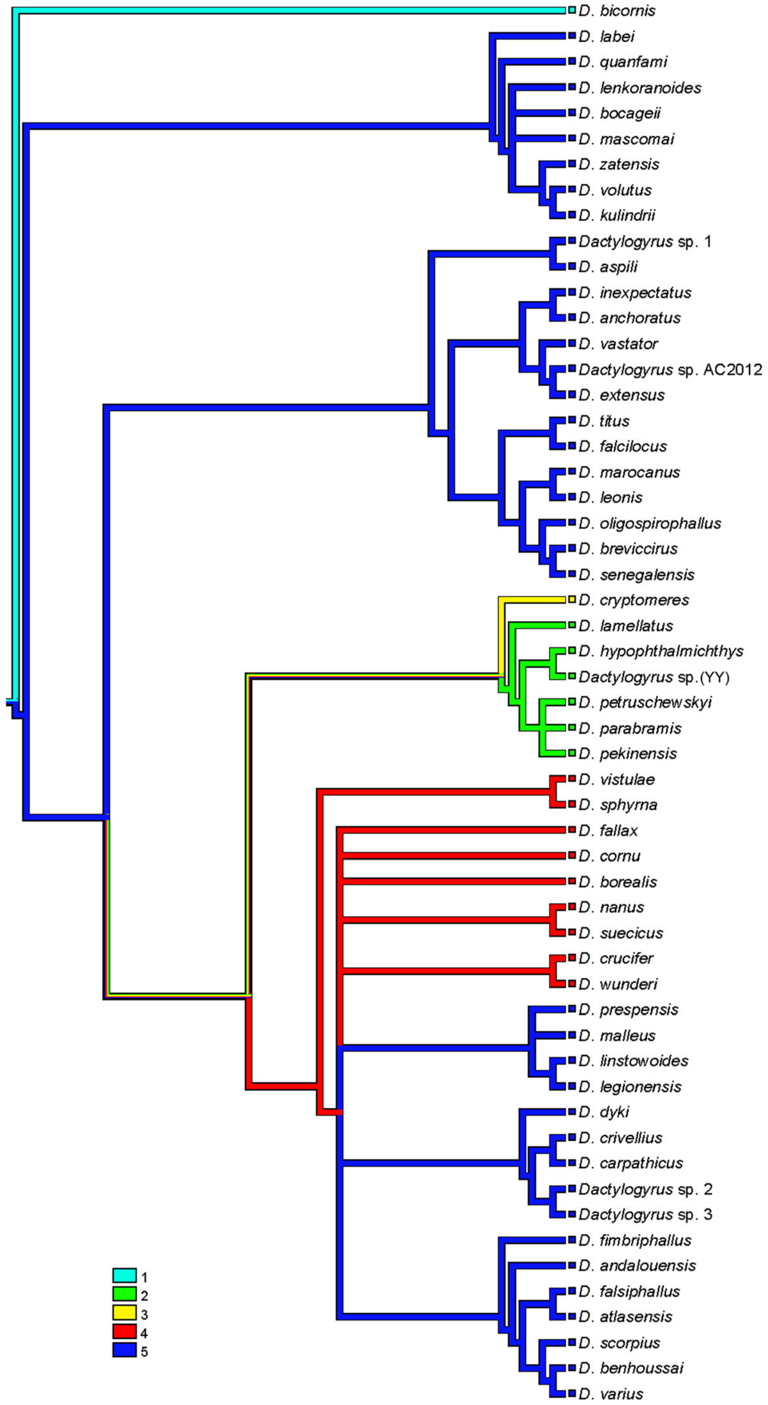

Fig. 3 Mapping of fish lineages into the BI reconstruction of *Dactylogyrus* phylogeny. Characters for fish lineages: 1, Acheilognathinae; 2, Xenocyprinae; 3, Gobioninae; 4, Leuciscinae; 5, Cyprininae

African *Dactylogyrus* parasitizing *Carasobarbus fritschii* (the tribe Torini within Cyprininae, see Fig. 5) are phylogenetically closely related to Asian *Dactylogyrus* species. *Dactylogyrus marocanus* is of African origin. Our mapping suggests that *D. marocanus* diverged within *Dactylogyrus* of African Labeonini and switched to Moroccan cyprinids (a morphologically and genetically identical form of this parasite was found in two species of Torini and two *Luciobarbus* species of Barbini). *Dactylogyrus* parasitizing Northwest African *Luciobarbus* are of European origin (Fig. 4). In addition, our analyses also showed the multiple origins of *Dactylogyrus* parasitizing Iberian *Luciobarbus*.

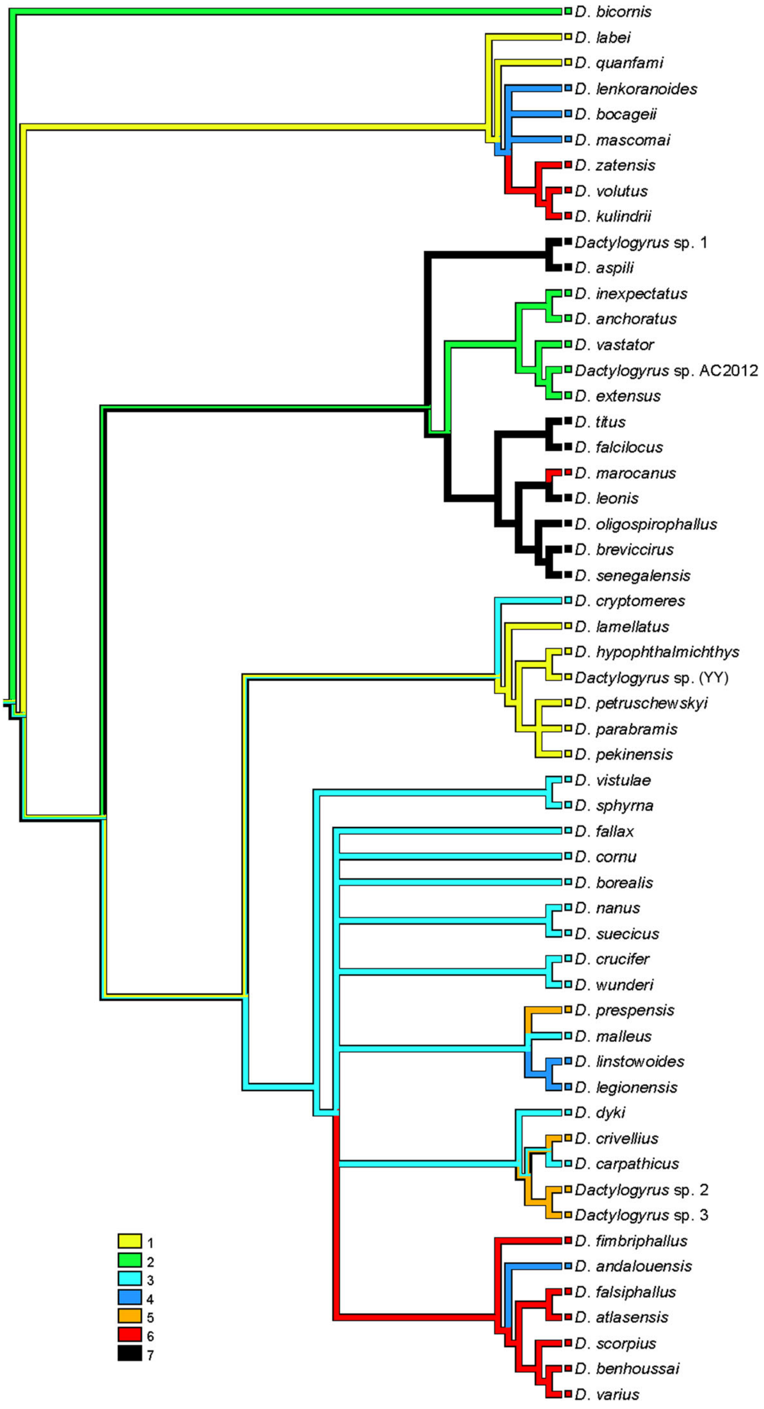

Fig. 4 Mapping of fish distribution onto the BI reconstruction of *Dactylogyrus* phylogeny. Characters for fish distribution: 1, South and Southeast Asia; 2, Eurasia; 3, Europe with West Asia; 4, Iberian Peninsula; 5, Balkan Peninsula; 6, Northwest Africa (Mediterranean Africa); 7, West Africa. Note: The area of introduction was not considered when fish distribution was evaluated. Concerning *C. gibelio*, it is not clear whether this species is native or introduced into Europe; therefore, we retained Eurasia

The mapping of fish distribution onto *Dactylogyrus* phylogeny demonstrated that one group of *Dactylogyrus* parasitizing Iberian *Luciobarbus* (i.e. *D. mascomai* El Gharbi, Renaud & Lambert, 1992, *D. lenkoranoides* El Gharbi, Renaud & Lambert, 1992 and *D. bocageii* Alvarez Pellitero, Simón Vicente & González Lanza, 1981) and the group of *Dactylogyrus* parasitizing Northwest African Torini probably originated from Asian cyprinids (most likely

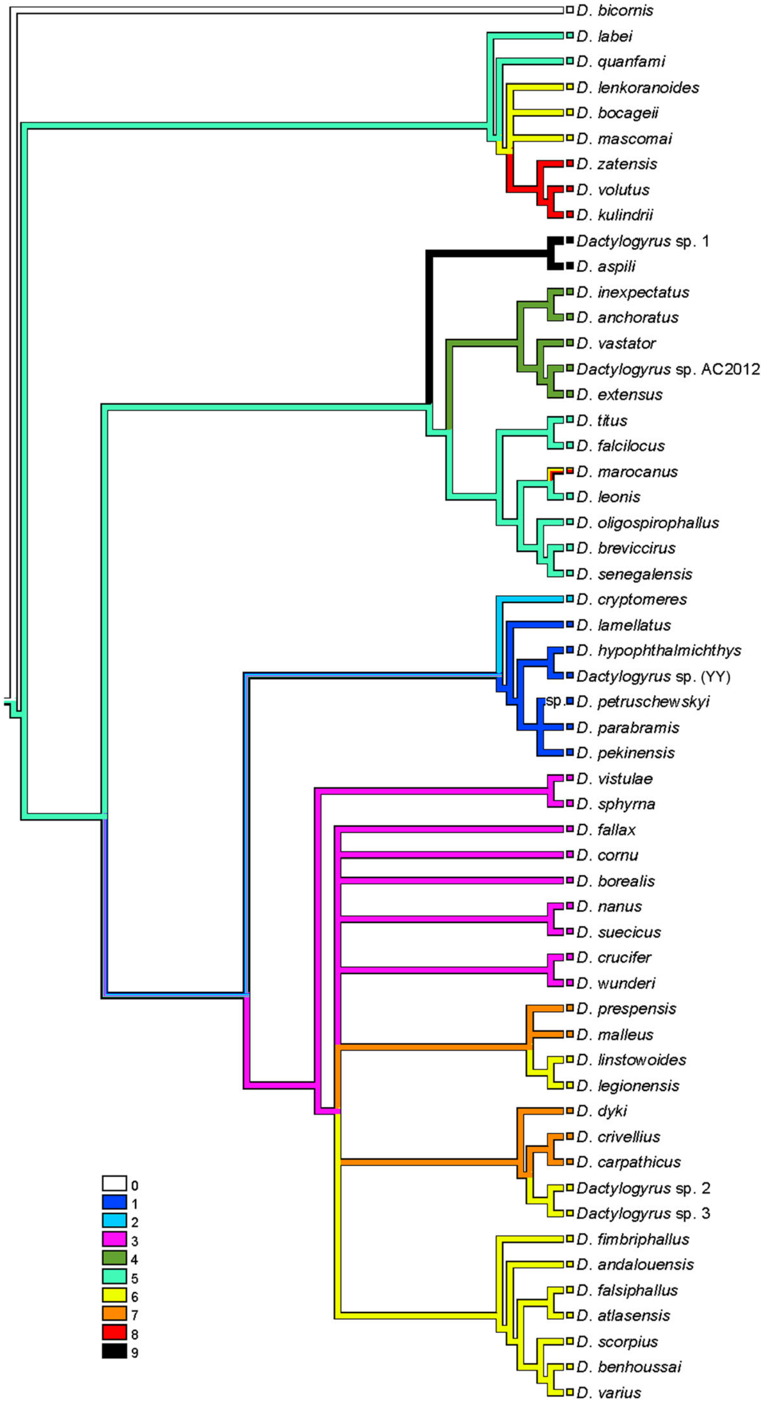

Fig. 5 Mapping of fish lineages including detailed branching of Cyprininae into the BI reconstruction of *Dactylogyrus* phylogeny. Characters for fish lineages: 0, Acheilognathinae; 1, Xenocyprinae; 2, Gobioninae; 3, Leuciscinae; 4, Cyprininae - Cyprinini; 5, Cyprininae - Labeonini; 6, Cyprininae - Barbini - *Luciobarbus*; 7, Cyprininae - Barbini - *Barbus*; 8, Cyprininae - Torini; 9, Cyprininae - Smiliogastrini

Labeonini). However, the other three *Dactylogyrus* of Iberian *Luciobarbus* are most probably of European origin. Whilst *D. linstowoides* El Gharbi, Renaud & Lambert, 1992 and *D. legionensis* González Lanza & Alvarez Pellitero, 1982 form the monophyletic group with the European *Dactylogyrus* of *Barbus* species, *D. andalousiensis* is included in the monophyletic group of Northwest African *Luciobarbus* species within *Dactylogyrus* of lineage III (i.e. the lineage including *Dactylogyrus* of Leuciscinae and some *Dactylogyrus* species of *Barbus*-*Luciobarbus* group).

Discussion

The present study was focused on host-specific monogeneans of *Dactylogyrus* as a potential tool for inferring historical contacts among their cyprinid hosts in the Mediterranean region, which is characterized by a high degree of endemism among cyprinid species. As indicated by a previous study [11, 12] and confirmed by our study, endemic Mediterranean cyprinids harbour endemic *Dactylogyrus* fauna. We investigated the origin of host-specific *Dactylogyrus* parasitizing Northwest African and Iberian cyprinid hosts, hypothesizing that phylogenetic relationships between *Dactylogyrus* species may cast new light on the biogeographical history of this fish group.

Šimková et al. [10] reconstructed the phylogeny of *Dactylogyrus* parasitizing central European cyprinid species (also including some invasive or introduced species). They presented evidence for three *Dactylogyrus* lineages in central Europe: the first includes *Dactylogyrus* of the Cyprininae (tribe Cyprinini), originating from Southeast Asia and historically introduced into Europe; the second includes *Dactylogyrus* of the Rasborinae and Xenocyprininae (the fish species of both groups originating from Southeast Asia and introduced into Europe) and the Gobioninae; and the last, a very diversified lineage, includes *Dactylogyrus* of the Leuciscinae and European *Barbus* (Barbini within Cyprininae). Šimková et al. [10] showed that the phylogenetic relationships between *Dactylogyrus* linages reflected the phylogenetic relationships between cyprinid lineages (recently represented by cyprinid subfamilies), except for the particular position of *Dactylogyrus* species parasitizing European *Barbus* species, which were nested within the highly diversified clade of *Dactylogyrus* parasitizing European Leuciscinae.

Herein, the phylogenetic position of *Dactylogyrus* parasitizing African cyprinids was evaluated for the first time. By our phylogenetic analyses, we showed that *Dactylogyrus* parasitizing the African cyprinids investigated in our study belong to three different lineages (I, II and III), which suggests their different origins and presumably also reflects the different histories of their cyprinid hosts. *Dactylogyrus* lineage II includes *Dactylogyrus* parasitizing the Cyprinini of Southeast Asian origin and West African Cyprininae (Labeonini investigated in our study), which suggests that West African cyprinids and their co-evolving *Dactylogyrus* originated from Asia (the basal position of *D. aspili* and *Dactylogyrus* sp. from small African *Enteromius* was not supported). This is in accordance with predictions on the origin of African cyprinid fauna [16, 25].

However, the situation concerning the origin of Northwest African cyprinids and their *Dactylogyrus* parasites is more complicated. *Dactylogyrus marocanus*, a single species infecting both Northwest African tribes of the Cyprininae, Torini and Barbini, was nested within *Dactylogyrus* lineage II. This parasite occurring on the representatives of two cyprinine lineages was previously reported in seven cyprinin species, mostly the representatives of Torini, by El Gharbi et al. [12] and also documented by our study. We showed a morphologically and genetically identical form of this species in *Carasobarbus fritschii*, *Pterocapoeta maroccana* Günther, 1902, *Luciobarbus ksibii* Boulanger, 1905 and *L. zayanensis* Doadrio, Casal-Lopéz & Yahyaoui, 2016. However, the abundance of *D. marocanus* was higher in two Torini species than in *Luciobarbus* species, suggesting that Torini are the main host species for its reproduction (see [9]). *Dactylogyrus marocanus* clusters within West African *Dactylogyrus* species parasitizing *Labeo* species, suggesting a single host-switch by *Dactylogyrus* to Northwest African Cyprininae from the group of Cyprininae achieving high diversification on the African continent. The attachment organ (haptor) of *D. marocanus* is of the same morphological type as that recognized for *Dactylogyrus* of West African *Labeo*, *Dactylogyrus* of small West African *Enteromius*, and two *Dactylogyrus* of Cyprinini of Southeast Asian origin i.e. *D. inexpectatus* Isjumova in Gussev, 1955 and *D. anchoratus* (Dujardin, 1845). In addition, *D. marocanus* is the only species with this type of haptor within the *Dactylogyrus* species parasitizing Northwest African cyprinids. This may suggest that haptor morphology, in this case, is a character shared by common ancestry. The similar morphology of the haptor in *Dactylogyrus* parasitizing phylogenetically closely related cyprinid species was previously demonstrated by Šimková et al. [9].

Our phylogenetic analyses using cyprinid-specific *Dactylogyrus* spp. confirmed the occurrence of different independent dispersal events from Asia (or Eurasia) to Africa concerning the Moroccan cyprinids belonging to hexaploid Torini (*Carasobarbus fritschii* and *Pterocapoeta maroccana* in our study) and tetraploid Barbini (*Luciobarbus* species), as was highlighted by the molecular phylogeny of cyprinid species [16, 17]. Middle East *Carasobarbus* and Northwest African *Carasobarbus* form a monophyletic group within the *Labeobarbus* clade, and *Pterocapoeta* occupies the basal position in this clade [16, 17]. Wang et al. [47] proposed that the group comprising the *Carasobarbus* lineage originated about 9.94 MYA in the Orient. The *Carasobarbus* lineage separated about 7.7 MYA. Tsigenopoulos et al. [17] dated the beginning of the diversification of the African hexaploid lineage to the Late Miocene following the closing of the seaway between the Mediterranean Sea and the Indian Ocean and the emergence of the Gomphotherium land bridge between Africa and Asia (the Arabian tectonic Plate) in the Middle Miocene. In the Tortonian stage, the Anatolian tectonic Plate (Asia Minor) was connected to the Arabian Plate to the east and was separated from Europe to the west, where the Aegean Sea formed [48]; this explains the absence of Torini in Europe [17]. The phylogenetic position of *Dactylogyrus* parasitizing *Carasobarbus fritschii* within *Dactylogyrus* lineage I and the phylogenetic affinity between *Dactylogyrus* species parasitizing South Asian Labeonini and *Dactylogyrus* species parasitizing Northwest African cyprinid species is in line with the hypothesis of the origin and historical dispersion of Northwest African Torini. The molecular phylogeny of tribes belonging to Cyprininae showed Labeonini to be a sister group to the group including other tribes with Torini in the basal position [16]. This may suggest close phylogenetic relationships between *Dactylogyrus* of Torini and Labeonini. However, our study suggests the need for future phylogenetic studies to investigate also the position of *Dactylogyrus* of Asian and African representatives of Torini as well as *Dactylogyrus* parasitizing other cyprinin tribes to specify the origin of *Dactylogyrus* diversity in Northwest African Torini.

Concerning the Mediterranean diversity of cyprinids, there are three main hypotheses of their historical dispersion explaining their actual distribution. All suggest that the cyprinids originated in Asia and reached the Mediterranean peninsulas via three main routes, a northern route [21], a southern route via land bridges connecting continents [25], and dispersion through the Mediterranean Sea during its supposed freshwater phase at the end of the Messinian [24]. According to the northern dispersal scenario, cyprinids dispersed slowly via river captures, through Siberia, and then from northern into southern Europe, from the late Oligocene until the late Pliocene (35–1.7 MYA). The colonization of southern Europe occurred before the alpine orogeny during the Miocene, which separated freshwater connections between northern and southern Europe [49]. Concerning *Luciobarbus*, it is hypothesized that they spread through central Europe to the Iberian Peninsula and Northwest Africa, and that, afterwards, a second invasion of *Barbus* from Asia colonized central Europe, where *Barbus* replaced *Luciobarbus* (except in the Iberian Peninsula due to the ancient isolation of the Iberian Peninsula from the rest of the European continent). This hypothesis was rejected for Iberian *Luciobarbus* by Zardoya & Doadrio [18]. According to the southern route hypothesis, cyprinids dispersed from Asia through Asia Minor via land bridges (Asian-Anatolian-Iranian, 33 MYA, and the Gomphotherium land bridge, 19 MYA) to the Balkans and Northern Africa, and subsequently to the Iberian Peninsula [24]. In accordance with this scenario, it is supposed that *Luciobarbus* colonized the Iberian Peninsula from Africa via southern Spain [18, 23]. The Lago Mare dispersal scenario [24] assumes that after the Messinian salinity crisis (5 MYA) the Mediterranean Sea underwent a lacustrine phase allowing the dispersion of freshwater fishes. This scenario predicts higher phylogenetic affinity among species in Mediterranean areas. Although this hypothesis is still widely cited, it has been largely discredited, both by geological evidence and phylogenetic studies (e.g. [19, 25]).

Recent views on the historical dispersion of *Luciobarbus* are, however, ambiguous. On the basis of morphological characters, Iberian and North African barbels are closely related to central European species, supporting the northern route of dispersion [21, 22], whilst molecular phylogenetic studies and a lack of fossil records of *Luciobarbus* in central Europe support the southern route of Iberian *Luciobarbus* dispersion [18, 19, 26]. Our phylogenetic reconstruction using host-specific *Dactylogyrus* would suggest that the northern route represents the more plausible scenario explaining the historical dispersion of *Luciobarbus* in Northwest Africa. This scenario is supported by our phylogenetic analyses, which indicate that (i) *Dactylogyrus* species parasitizing Northwest African *Luciobarbus* have a clearly European origin, and (ii) the monophyletic group of *Dactylogyrus* including *D. balistae* Simón Vicente, 1981, *D. legionensis* and *D. linstowoides* parasitizing Iberian *Luciobarbus* form a well-supported clade with *Dactylogyrus* parasitizing European *Barbus*. In addition, the Iberian species *D. andalousiensis* occupies the basal position in the clade including the monophyletic group of *Dactylogyrus* species parasitizing Northwest African *Luciobarbus* (a finding well supported by BP and PP using the combined data of partial 18S rDNA and ITS1). Even though our sampling of *Dactylogyrus* parasites did not include *Dactylogyrus* representatives of Middle East cyprinids, we showed that *Dactylogyrus* species parasitizing Northwest African *Luciobarbus*, four of the *Dactylogyrus* species parasitizing Iberian *Luciobarbus*, *Dactylogyrus* species parasitizing Greek *Luciobarbus*, *Dactylogyrus* species parasitizing Balkan *Barbus*, and *Dactylogyrus* species parasitizing the widely distributed European *Barbus barbus* form together with *Dactylogyrus* parasitizing Leuciscinae the well-supported lineage III. This may suggest the common origin of *Dactylogyrus* parasitizing *Luciobarbus*/*Barbus* (Cyprininae) of different Mediterranean areas and *Dactylogyrus* of European Leuciscinae. Indubitably, there is a strong relationship between *Dactylogyrus* parasitizing Northwest African *Luciobarbus* (Barbini) and those parasitizing European cyprinids belonging to the subfamily Leuciscinae and the tribe Barbini of the subfamily Cyprininae, identified in our *Dactylogyrus* lineage III. However, in this case, there is a large discrepancy between the phylogenies of the hosts and *Dactylogyrus* parasites, and the relationships in lineage III rather point to historical host-switching events.

In our study, we showed the close phylogenetic relationships between (i) *Dactylogyrus* parasitizing Northwest African Torini and one group of *Dactylogyrus* parasitizing Iberian *Luciobarbus* species and (ii) *Dactylogyrus* parasitizing Northwest African Barbini and the second group of *Dactylogyrus* parasitizing also Iberian *Luciobarbus* species. This revealed (i) multiple historical contacts between Iberian *Luciobarbus* and two different lineages of Northwest African cyprinids with different origins and historical dispersions, and subsequently (ii) two independent diversifications of *Dactylogyrus* in Iberian *Luciobarbus*. The exchange of fauna between the Iberian Peninsula and Northwest Africa is hypothesized for the beginning of the Messinian salinity crisis 5.96 MYA [50], which was initiated by the closing of the Betic and Rifian corridors in Spain and Morocco [51–53]. If this event was responsible for the common origin of *Dactylogyrus* parasitizing Iberian and Northwest African cyprinids, the origin and diversification of Iberian *Luciobarbus* seem to be older than predicted by the Lago Mare route of *Luciobarbus* dispersion. Mesquita et al. [54] suggested an even earlier differentiation of Mediterranean *Luciobarbus* lineages (7.3 MYA). At the end of the Messinian 5.33 MYA, all connections between North African and Iberian populations were closed by the formation of the Strait of Gibraltar [52]. However, Machordom & Doadrio [19] suggested that the Betic area was connected with the Kabilian Mountains after its isolation from the Rifian area by the Betic-Kabilian plate in the Pliocene (3.3 MYA). Cahuzac et al. [55] proposed the existence of plates also between southern Spain and the Maghreb. These plates may potentially have served as the contact zones between Iberian and North African cyprinids and may alternatively have contributed to the common ancestry of *Dactylogyrus* parasitizing Iberian *Luciobarbus* and Northwest African Torini or Barbini. However, we failed to identify any reliable resource documenting historical contacts between Iberian *Luciobarbus* and the two Moroccan cyprinid lineages.

Mesquita et al. [54] identified three polytomic evolutionary lineages of Iberian *Luciobarbus*, potentially suggesting multiple speciation events which could likely explain the evidence of two lineages for Iberian *Dactylogyrus*. However, the different positions of the two Iberian *Dactylogyrus* lineages in the phylogenetic tree have no association with the evolution and recent distribution of Iberian *Luciobarbus* (according to [54]), *L. bocagei* (Steindachner, 1864) representing the Atlantic lineage, *L. guiraonis* (Steindachner, 1866) representing the Mediterranean lineage, and *L. sclateri* representing the South-Western and South-Eastern lineage. *Dactylogyrus bocageii*, previously reported as a species endemic to Spanish *Luciobarbus* [11], was present in all three Iberian *Luciobarbus* species investigated in our study and living recently in allopatry. As indicated before, *D. andalousiensis* is a single Iberian *Dactylogyrus* species with the basal position in the clade including the monophyletic group of *Dactylogyrus* species parasitizing the Northwest African *Luciobarbus*. This parasite was previously recorded on two *Luciobarbus* species, namely *L. sclateri* and *L. microcephalus* (Almaça, 1967), both restricted to southern Portugal and Spain [11], but representing different evolutionary lineages [54].

Conclusions

To our knowledge, this study is the first to investigate the origin and phylogenetic position of Northwest African and Iberian *Dactylogyrus*, monogenean parasites specific to cyprinid fish. The phylogenetic reconstruction of these host-specific monogeneans sheds new light on historical contacts between African and European (here Iberian) cyprinids, these contacts associated with host switches of *Dactylogyrus* parasites. More specifically, phylogenetic analyses using *Dactylogyrus* demonstrated different and independent dispersal events from Asia (or Eurasia) to Africa concerning two lineages of Moroccan cyprinids: (i) *Carasobarbus fritschii* and *Pterocapoeta maroccana* belonging to hexaploid Torini, and (ii) *Luciobarbus* species belonging to teptraploid Barbini. In addition, our study revealed that *Dactylogyrus* parasitizing Iberian *Luciobarbus* do not form a monophyletic group, i.e. we demonstrated close phylogenetic relationships between (i) *Dactylogyrus* parasitizing Northwest African Torini and one group of *Dactylogyrus* parasitizing Iberian *Luciobarbus* species, and (ii) *Dactylogyrus* parasitizing Northwest African Barbini and the second group of *Dactylogyrus* parasitizing also Iberian *Luciobarbus*. This suggests multiple historical contacts between Iberian *Luciobarbus* and Northwest African cyprinids with different origins and historical dispersions, and subsequently two independent diversification of *Dactylogyrus* in Iberian *Luciobarbus*.

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Availability of data and materials

The data supporting the conclusions of this study are included in this article. The newly generated sequences were submitted to the GenBank database under the accession numbers shown in Table 1.

Authors' contributions

AŠ conceived the ideas and designed the study. AŠ, MB, IR and JV conducted the fieldwork and collected the data. AŠ, MB and IR analysed the data. AŠ wrote the manuscript with assistance from JV. All authors read and approved the final manuscript.

Ethics approval

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).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic. [2]Laboratory of Biodiversity, Ecology and Genome, Faculty of Sciences, Mohammed V University in Rabat, Ibn Batouta 4, 1014 RP Rabat, Morocco. [3]Department of Ecology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic.

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PAPER III

The phylogenetic relationships and species richness of host-specific *Dactylogyrus* parasites shaped by the biogeography of Balkan cyprinids

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The phylogenetic relationships and species richness of host-specific *Dactylogyrus* parasites shaped by the biogeography of Balkan cyprinids

Michal Benovics[1], Yves Desdevises[2], Jasna Vukić[3], Radek Šanda[4] & Andrea Šimková[1]

Parasites exhibiting a high degree of host specificity are expected to be intimately associated with their hosts. Therefore, the evolution of host-specific parasites is at least partially shaped by the evolutionary history and distribution of such hosts. Gill ectoparasites of *Dactylogyrus* (Monogenea) are specific to cyprinid fish. In the present study, we investigated the evolutionary history of 47 *Dactylogyrus* species from the Balkan Peninsula, the Mediteranean region exhibiting the highest cyprinid diversity in Europe, and from central European cyprinids. Phylogenetic analyses revealed four well-supported clades of endemic and non-endemic *Dactylogyrus* spp. with four basal taxa. Endemic cyprinids with a limited distribution range were parasitized by endemic *Dactylogyrus* species, but some of them shared several *Dactylogyrus* species with central European cyprinids. Species delimitation analyses based on molecular data suggest that *Dactylogyrus* diversity is higher than that defined from morphology. Some endemic cyprinid species harboured *Dactylogyrus* species of different origins, this probably resulting from multiple host switching. Our results support the view that the evolution of *Dactylogyrus* in the Balkans has been influenced not only by the historical dispersion and distribution of their cyprinid hosts, but also by recent contacts of non-native cyprinid species with endemic cyprinid fauna in this region.

The species richness of parasitic taxa and their distribution in host species is usually closely related to the history, dispersion and diversity of their hosts[1–3]. The parasitic genus *Dactylogyrus* (Monogenea), known for its wide species richness (over 900 nominal species according to Gibson *et al.*[4]), is restricted mainly to fish species of Cyprinidae, a highly diversified group of primarily freshwater fish[5]. *Dactylogyrus* species exhibit a high degree of host specificity within the multitude of their host species[6].

Previous studies suggest that each cyprinid species can host at least one *Dactylogyrus* species[7–9]. Within one host species the distribution of *Dactylogyrus* species is restricted to specific microhabitats, i.e. different *Dactylogyrus* species occupy distinct niches within host gills[10–12]. The evolution of niche preference is linked with changes of at least one parameter determining niche position on fish gills (e.g. the changes in the positions among the different gill arches or different segments of a given gill arch)[6]. It has been hypothesized that, over evolutionary time, monogeneans developed copulatory organs of different shapes and sizes, which resulted in reproductive isolation within overlapping microhabitats[13]. This was previously documented in *Dactylogyrus* species as well[14].

Unlike central and northern Europe, where the cyprinid fauna is relatively uniform, southern European peninsulas are extremely rich in endemic cyprinid species[15]. The endemic cyprinid fauna of Mediterranean regions consists of several highly diversified genera whose origin and historical biogeography are still poorly known in spite of several recent studies[16–20]. Zardoya *et al.*[21] investigated 15 lineages (52 species) of Greek cyprinids

[1]Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 61137, Brno, Czech Republic. [2]Sorbonne Universités, UPMC Univ Paris 06, CNRS, Biologie Intégrative des Organismes Marins (BIOM), Observatoire Océanologique de Banyuls/Mer, F-66650, Banyuls/Mer, France. [3]Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44, Prague, Czech Republic. [4]National Museum, Václavské Náměstí 68, 115 79, Prague, Czech Republic. Correspondence and requests for materials should be addressed to M.B. (email: benovics@mail.muni.cz)

and proposed that species related to Danubian cyprinid fauna colonized the Balkan Peninsula during two different time periods. The first one occurred during the Miocene, when fish species such are *Barbus cyclolepis*[22], *Alburnoides strymonicus*[19], *Telestes beoticus*, *T. pleurobipunctatus*[20], and *Squalius peloponensis*[18] diverged. These species show relatively high molecular divergence in comparison to central European sister group taxa. The second period is related to the Plio-Pleistocene connection of the Balkan Peninsula and the River Danube via river captures[23,24]. This dispersion event included species such are *Barbus balcanicus*[25], *Squalius vardarensis* and species of *Chondrostoma* and *Alburnus* genera[26], which exhibit a much lower degree of molecular divergence with respect to Danubian-related taxa. Previous studies on the phylogeny of Balkan cyprinids are focused on *Squalius*[18,26–30], which is one of two genera (with *Barbus*) inhabiting all three southern European peninsulas. According to the above-cited study by Sanjur *et al.*[30], based on analysis of the mitochondrial cytochrome *b* gene, Balkan *Squalius* species are grouped into three major clades. Several studies, based on different molecular markers and the analysis of several morphological traits, suggested that the Balkan *Squalius* species with the greatest ancestral diversification is *Squalius keadicus*, which split from other *Squalius* lineages approximately 9 Mya[24,26]. The Balkan ancient lake system, known as Dessaretes, emerged in the Pliocene, and was suggested to have play an important role in freshwater biota speciation processes. For this reason, it is considered to have been a hotspot of endemic Balkan biodiversity[31–35]. The Dessaretes lake system formerly included Lake Ohrid (located in Albania and F.Y.R.O.M.), Lake Prespa (Albania, Greece, F.Y.R.O.M.), Lake Mikri Prespa (Albania, Greece) and Lake Maliq (Albania). Recently, the current distribution of many cyprinid species from the "Dessaretes" region was reevaluated. For example, *Barbus prespensis*, initially known as an endemic species from Lake Prespa, was recently shown to be widespread in the south-eastern Adriatic basin, together with other presumably endemic species from Lake Prespa, namely *Alburnoides prespensis* and *Squalius prespensis*[19,25,36]. This basin is a part of the evaporated Lake Maliq, historically connected to Lake Prespa and drained after the Second World War[33].

Gregory[37] suggested that hosts with a larger area of distribution are infected by more parasitic species. Concerning cyprinids, widely distributed species across Europe such as *Rutilus rutilus* and *Squalius cephalus* harbour up to 9 *Dactylogyrus* species[11,38]. In contrast, Dupont and Lambert[7] found only 5 *Dactylogyrus* species on *Rutilus rubilio*, an endemic cyprinid species in the Apennine Peninsula. A phylogenetic reconstruction including 51 *Dactylogyrus* species and based on molecular data suggested that species parasitizing central European cyprinids form three monophyletic groups[11] and are associated with different phylogenetic lineages of cyprinid species representing subfamilies with different origins, histories, and biogeographical distributions. Since studies of endemic and non-endemic *Dactylogyrus* from Balkan cyprinids are scarce and mainly based on morphological data[7,39–41], the evolutionary histories and patterns of endemism of these host-specific species are still unresolved. Several previous studies concerning different regions of the northern Mediterranean Sea suggested that endemic cyprinids harbour endemic *Dactylogyrus* species[7,9,42]. Some phylogenetic studies were focused on *Dactylogyrus* species from selected cyprinid genera, such as *Dactylogyrus* spp. parasitizing *Barbus* species[43]. According to the authors, such *Dactylogyrus* species are supposed to exhibit both genetic and morphological variabilities between different host species. Dupont[44] investigated the historical biogeography of *Dactylogyrus* species of endemic *Rutilus, Luciobarbus*, and *Pachychilon* hosts from the Balkan Peninsula and suggested that the endemism of *Dactylogyrus* can be explained by the formation of landmass and freshwater streams during the Neogene and Pleistocene eras.

The aim of the present study was to investigate the diversity, evolutionary history, and phylogenetic relationships of *Dactylogyrus* spp. parasitizing endemic cyprinids of the Balkan Peninsula. First, we analyzed the degree of endemism in *Dactylogyrus* species parasitizing these cyprinids. Next, we focused on the phylogenetic relationships between endemic *Dactylogyrus* and commonly distributed *Dactylogyrus* (species shared between central European and endemic Balkan cyprinid species) in order to infer potential scenarios of historical contact between different cyprinids. Concerning *Dactylogyrus* species with a wide host range, we also searched for genetic structuration by analyzing the level of genetic diversity and its correlation with the geographical distances between their hosts. Finally, we assessed the species status of generalist *Dactylogyrus* on the basis of molecular data in order to test whether the degree of genetic variability was in concordance with the current species status based on a classical taxonomical approach.

Results

Dactylogyrus species richness. A total of 53 *Dactylogyrus* species were identified from cyprinid hosts from the Balkans (Table 1) and central Europe. 47 species were collected from endemic Balkan cyprinids. Six additional species were collected from the Czech Republic and included in analyses. Balkan cyprinids were parasitized by 1 to 5 *Dactylogyrus* species with an average of 2 species per host species. The highest *Dactylogyrus* species diversity was reported on representatives of the genera *Pachychilon* – *P. pictum* (5); *Squalius* – *S. squalus* (4) and *S. prespensis* (4); *Barbus* – *B. prespensis* (4); and *Rutilus* – *R. basak* (4), *R. lacustris* (4), and *R. ohridanus* (4). Eight *Dactylogyrus* species were unidentified and are expected to be new to science. These potentially new species were collected from the following host species: *Delminichthys adspersus*, *Chondrostoma knerii*, *Squalius tenellus, Luciobarbus albanicus, L. graecus, Tropidophoxinellus spartiaticus, Telestes karsticus* and *Pachychilon macedonicum*.

Phylogenetic analyses and genetic distances. The concatenated sequence alignment of partial 18S and partial 28S rDNA from representatives of 54 *Dactylogyrus* species from the Balkan Peninsula and central Europe contained 1158 unambiguous nucleotide positions. The data were treated as partitioned and GTR+I was selected as the most optimal evolutionary model for the 446 bp-long partial 18S rDNA sequences, and GTR+I+G for the 712 bp-long partial 28S rDNA sequences. BI (Bayesian inference) and ML (Maximum Likelihood) analyses produced trees with identical topologies which varied in node support values (Fig. 1). The resulting phylogram divided most of the species into 4 strongly-to-moderately supported clades. Four *Dactylogyrus* species

***Dactylogyrus* species**	**Host**	**Locality**	**partial 18S + ITS1**	**partial 28S**
D. auriculatus	*Abramis brama*	CZ1	MG792838*	MG792952*
D. alatus	*Alburnus neretvae*	B1	MG792842*	MG792956*
	Alburnus neretvae	B2	MG792843*	MG792957*
D. anchoratus	*Carassius gibelio*	C2	KY859795	KY863555
D. balkanicus	*Barbus plebejus*	C1	MG792861*	MG792976*
	Barbus prespensis	G1	KY201093	KY201107
	Barbus rebeli	A6	MG795863*	MG792978*
D. borealis	*Phoxinus* sp.	B9	KY629343	KY629372
D. caballeroi	*Rutilus ohridanus*	A4	MG792902*	MG793018*
	Rutilus rutilus	CZ1	AJ564114	MG793022*
D. carpathicus	*Barbus barbus*	CZ1	KY201098	KY201111
D. caucasicus	*Alburnoides devoli*	A1	MG792840*	MG792954*
	Alburnoides fangfangae	A2	MG792841*	MG792955*
	Alburnoides prespensis	G1	MG792847*	MG792961*
D. cornu	*Vimba vimba*	CZ1	KY629342	KY629371
D. crivellius	*Barbus balcanicus*	G4	MG792854*	MG792969*
	Barbus peloponnesius	G7	KY629339	KY629368
	Barbus plebejus	C1	MG792862*	MG792977*
	Barbus prespensis	G1	KY201094	KY201108
	Barbus rebeli	A6	MG792863*	MG792979*
	Barbus sp.	A7	MG792866*	MG792981*
D. crucifer	*Rutilus lacustris*	G12	MG792898*	MG793014*
	Rutilus rutilus	CZ1	AJ564120	KY629374
D. difformis	*Scardinius plotizza*	B4	MG792908*	MG793025*
D. difformoides	*Scardinius plotizza*	B4	MG792909*	MG793026*
D. dirigerus	*Chondrostoma ohridana*	G1	MG792873*	MG792988*
	Chondrostoma vardarensis	G2	MG792876*	MG792991*
	Chondrostoma vardarensis	G3	MG792877*	MG792992*
D. dyki	*Barbus balcanicus*	G4	MG792855*	MG792970*
	Barbus barbus	CZ1	KY629338	KY629367
	Barbus cyclolepis	G5	MG792856*	MG792971*
	Barbus peloponnesius	G6	MG792858*	MG792973*
	Barbus peloponnesius	G7	MG792859*	MG792974*
	Barbus prespensis	A5	KY201095	KY201109
	Barbus prespensis	G1	KY859804	KY859803
	Barbus rebeli	A6	MG792865*	MG792980*
	Barbus sperchiensis	G8	MG792867*	MG792982*
	Barbus strumicae	G1	MG792868*	MG792983*
D. ergensi	*Chondrostoma knerii*	B4	MG792870*	MG792985*
	Chondrostoma ohridana	G1	MG792874*	MG792989*
	Chondrostoma vardarensis	G2	MG792878*	MG792993*
D. erhardovae	*Rutilus aula*	C2	MG792893*	MG793009*
	Rutilus basak	B10	MG792894*	MG793010*
D. extensus	*Cyprinus carpio*	—	KM277459	AY553629
D. fallax	*Chondrostoma nasus*	CZ1	MG792872*	MG792987*
	Rutilus rutilus	CZ1	MG792906*	MG793023*
	Vimba vimba	CZ1	KY629341	KY629370
D. folkmanovae	*Squalius cephalus*	CZ1	MG792912*	MG793029*
	Squalius cephalus	B7	MG792911*	MG793028*
	Squalius orpheus	G9	MG792916*	MG793035*
	Squalius platyceps	A8	MG792919*	MG793038*
	Squalius prespensis	A9	MG792921*	MG793040*
	Squalius prespensis	G1	MG792922*	MG793041*
	Squalius sp.	G10	MG792926*	MG793032*
	Squalius squalus	C4	MG792928*	MG793044*
	Squalius vardarensis	G4	MG792935*	MG793049*

Continued

***Dactylogyrus* species**	**Host**	**Locality**	**partial 18S + ITS1**	**partial 28S**
D. formosus	*Carassius gibelio*	C2	MG792869*	MG792984*
D. ivanovichi	*Pachychilon pictum*	G1	MG792883*	MG792999*
D. izjumovae	*Scardinius dergle*	C1	MG792907*	MG793024*
	Scardinius plotizza	B4	MG792910*	MG793027*
D. malleus	*Barbus barbus*	CZ1	KY201099	KY201112
D. martinovici	*Pachychilon pictum*	A8	MG792884*	MG793000*
	Pachychilon pictum	G1	MG792885*	MG793001*
D. minor	*Alburnus scoranza*	A4	MG792848*	MG792962*
D. nanoides	*Squalius cephalus*	B7	MG792913*	MG793030*
	Squalius prespensis	G1	MG792923*	MG793045*
	Squalius squalus	B11	MG792929*	MG793046*
D. omenti	*Aulopyge huegelii*	B3	KY201091	KY201105
D. parvus	*Alburnus scoranza*	A4	MG792849*	MG792963*
D. petenyi	*Barbus balcanicus*	G4	KY201097	KY201113
	Barbus cyclolepis	G5	MG792857*	MG792972*
	Barbus peloponnesius	G7	MG792860*	MG792975*
D. petkovici	*Pachychilon pictum*	A8	MG792886*	MG793002*
	Pachychilon pictum	G1	MG792887*	MG793003*
D. prespensis	*Barbus prespensis*	G1	KY201096	KY201110
D. prostae	*Squalius cephalus*	CZ1	MG792914*	MG793031*
	Squalius pamvoticus	G13	MG792917*	MG793036*
	Squalius prespensis	G1	MG792924*	MG793042*
	Squalius sp.	G10	MG792927*	MG793033*
D. rarissimus	*Alburnus neretvae*	B1	MG792844*	MG792958*
	Alburnus neretvae	B2	MG792845*	MG792959*
	Pelasgus laconicus	G11	MG792890*	MG793006*
	Rutilus basak	B10	MG792895*	MG793011*
	Rutilus lacustris	G12	MG792899*	MG793015*
	Rutilus ohridanus	A4	MG792903*	MG793019*
	Telestes alfiensis	G15	MG792938*	MG793055*
	Telestes dabar	B12	MG792939*	MG793056*
	Telestes fontinalis	C6	MG792940*	MG792997*
	Telestes metohiensis	B13	MG792944*	MG793059*
D. rosickyi	*Pachychilon pictum*	G1	MG792888*	MG793004*
D. rutili	*Rutilus basak*	B10	MG792896*	MG793012*
	Rutilus lacustris	G12	MG792900*	MG793016*
	Rutilus ohridanus	A4	MG792904*	MG793020*
D. rysavyi	*Alburnoides thessalicus*	G3	MG792851*	MG792965*
D. sekulovici	*Pachychilon pictum*	G1	MG792889*	MG793005*
D. soufii	*Telestes montenigrinus*	A10	MG792946*	MG793061*
Dactylogyrus sp. 1	*Squalius tenellus*	B5	MG792933*	MG793050*
Dactylogyrus sp. 2	*Luciobarbus graecus*	G8	KY201101	KY201115
Dactylogyrus sp. 3	*Luciobarbus albanicus*	G10	KY201100	KY201114
Dactylogyrus sp. 4	*Delminichthys adspersus*	B6	MG792881*	MG792995*
Dactylogyrus sp. 5	*Pachychilon macedonicum*	G3	MG792882*	MG792998*
Dactylogyrus sp. 6	*Tropidophoxinellus spartiaticus*	G6	MG792950*	MG793065*
Dactylogyrus sp. 7	*Chondrostoma knerii*	B4	MG792871*	MG792986*
Dactylogyrus sp. 8	*Telestes karsticus*	C7	MG792942*	MG793057*
D. sphyrna	*Rutilus basak*	B10	MG792897*	MG793013*
	Rutilus ohridanus	A4	MG792905*	MG793021*
	Vimba vimba	CZ1	MG792951*	MG793066*
D. suecicus	*Rutilus lacustris*	G12	MG792901*	MG793017*
	Telestes montenigrinus	A10	MG792947*	MG793062*
D. tissensis	*Alburnoides thessalicus*	G3	MG792852*	MG792966*
D. vastator	*Aulopyge huegelii*	B3	KY201092	KY201106
	Carassius gibelio	CZ2	KY201103	KY629366

Continued

Dactylogyrus species	Host	Locality	partial 18S + ITS1	partial 28S
D. vistulae	*Alburnoides ohridanus*	A3	MG792846*	MG792960*
	Alburnoides strymonicus	G2	MG792850*	MG792964*
	Alburnoides thessalicus	G3	MG792853*	MG792968*
	Chondrostoma ohridana	G1	MG792875*	MG792990*
	Chondrostoma phoxinus	B5	MG792880*	MG792994*
	Chondrostoma vardarensis	G3	MG792879*	MG792967*
	Phoxinellus alepidotus	B7	MG792891*	MG793007*
	Phoxinellus pseudalepidotus	B8	MG792892*	MG793008*
	Squalius illyricus	C3	MG792915*	MG793034*
	Squalius peloponensis	G14	MG792918*	MG793037*
	Squalius platyceps	A8	MG792920*	MG793039*
	Squalius prespensis	A9	KY629340	KY629369
	Squalius prespensis	G1	MG792925*	MG793043*
	Squalius squalus	B11	MG792930*	MG793047*
	Squalius svallize	C5	MG792932*	MG793049*
	Squalius tenellus	B5	MG792934*	MG793051*
	Squalius vardarensis	G4	MG792936*	MG793053*
	Telestes fontinalis	C6	MG792941*	MG792996*
	Telestes karsticus	C7	MG792943*	MG793058*
	Telestes metohiensis	B13	MG792945*	MG793060*
	Telestes montenigrinus	A10	MG792948*	MG793063*
	Telestes pleurobipunctatus	G7	MG792949*	MG793064*
D. vranoviensis	*Squalius squalus*	B11	MG792931*	MG793048*
	Squalius vardarensis	G4	MG792937*	MG793054*
D. zandti	*Abramis brama*	CZ1	MG792839*	MG792953*

Table 1. List of collected *Dactylogyrus* species and their cyprinid host species. GenBank accession numbers are included. New sequences obtained in this study are marked by asterisks (*).

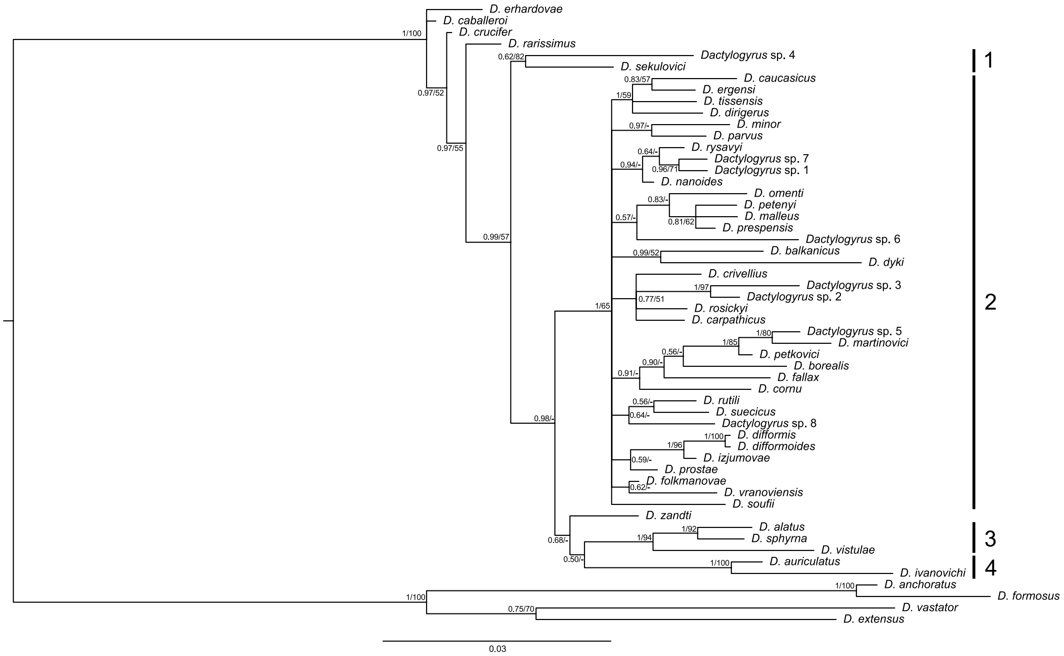

Figure 1. *Phylogram of 54 Dactylogyrus species from the Balkans and Central Europe reconstructed by Bayesian inference.* The tree is based on concatenated data of partial 18S rDNA and partial 28S rDNA sequences. Values along branches indicate posterior probabilities and boostrap values resulting from Bayesian inference and Maximum likelihood analyses, respectively. Values <0.80 for BI and <50% for ML are indicated by dashes (-). Branch lengths correspond to the expected number of substitutions per site. Labels 1–4 refer to different *Dactylogyrus* lineages. The phylogenetic tree was rooted using *Dactylogyrus* species parasitising *Carassius gibelio* and *Cyprinus carpio* (following Šimková *et al.*[12]).

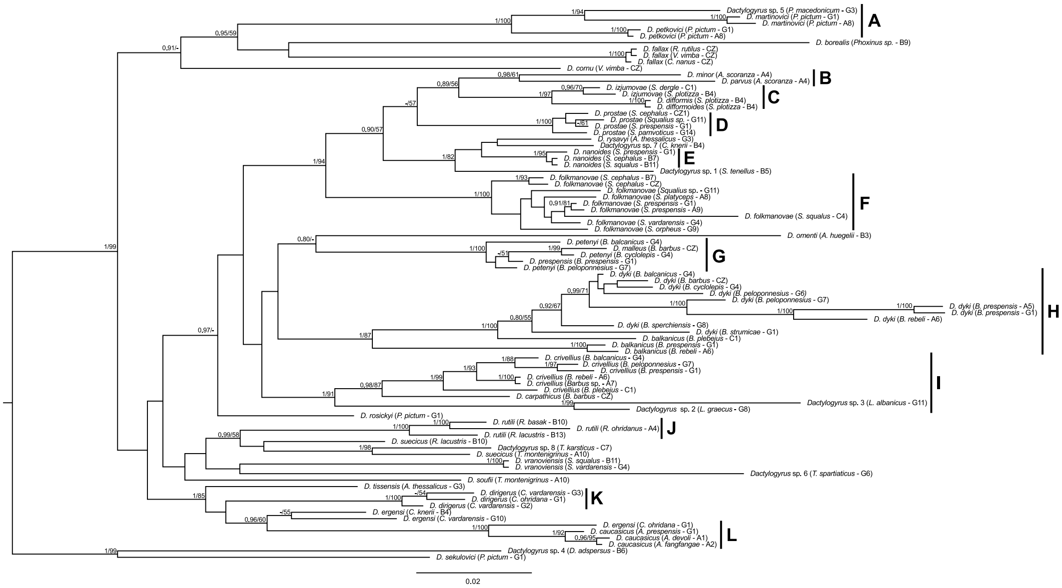

Figure 2. *Phylogram of selected Dactylogyrus species from the Balkans and Central Europe constructed by Bayesian inference*. The tree is based on concatenated data of partial 18S rDNA, ITS1 region and partial 28S rDNA sequences. Values along branches indicate posterior probabilities and boostrap values resulting from Bayesian inference and maximum likelihood analyses, respectively. Values <0.80 for BI and <50% for ML are indicated by dashes (-). Branch lengths correspond to number of substitutions per site. Labels A–L refer to different, well supported, *Dactylogyrus* clades.

(*D. erhardovae*, *D. caballeroi*, *D. crucifer* and *D. rarissimus*) were placed in an external position to these four clades. The first clade (clade 1), weakly supported by BI and well supported by ML analyses, included the species *D. sekulovici* from *Pachychilon pictum* and *Dactylogyrus* sp. 4 from *Delminichthys adspersus*. The second clade (clade 2), highly supported by BI and weakly supported by ML analyses, was the largest and included all species parasitizing *Barbus* and *Luciobarbus*. *Dactylogyrus* species endemic for the Balkan Peninsula and also widely distributed *Dactylogyrus* species clustered in this second clade. Generally, species with similarly shaped haptoral hard parts clustered together and such clusters were well or moderately supported by at least BI analysis (PP, posterior probability > 0.81). For example, *D. petkovici*, *D. martinovici* and *Dactylogyrus* sp. 5, representing a monophyletic group, share a similar type of thin anchor hooks and a ventral bar with five extremities, while *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3, representing another monophyletic group, display hard parts of the haptor that are almost indistinguishable in shape. Three *Dactylogyrus* species from *Barbus* (i.e. *D. petenyi*, *D. malleus* and *D. prespensis*, which also share a similar shape of their haptoral hard parts) were clustered with *D. omenti* from *Aulopyge huegelii*. The third clade was strongly supported by both BI and ML analyses and included *D. alatus*, *D. sphyrna* and *D. vistulae*, which are large worms with large haptoral anchor hooks. The last well-supported clade (PP = 1, BS, bootstrap value = 100) included *D. auriculatus* from *Abramis brama* and *D. ivanovichi* from *P. pictum* (clade 4), which exhibited identically shaped MCO (male copulatory organ) hard parts but VA (vaginal armament) of slightly different shape. All species from clades 3 and 4, except *D. alatus*, had no connective ventral bar. *Dactylogyrus zandti* appeared to be a sister species to clades 3 and 4, but its position was not supported.

To resolve the phylogenetic relationships among groups within the second clade, we used a concatenated alignment of partial 18S, 28S rDNA, and the highly variable ITS1 (Internal Transcribe Spacer 1) region. The alignment of 86 sequences comprised 1503 unambiguously aligned nucleotide positions.The most optimal evolutionary models were TrNef+I for the alignment of 446 bp-long partial 18S rDNA sequences, SYM+G for the alignment of 344 bp-long ITS1 sequences, and TVMef+I+G for the alignment of 713 bp-long partial 28S rDNA sequences. BI and ML analyses generated trees with the same topologies (Fig. 2). The resulting trees were rooted using clade 1 from the first phylogenetic reconstruction (Fig. 1).

The phylogenetic analyses divided clade 2 into several strongly-to-moderately supported groups. Group A included species parasitizing *Pachychilon*, these sharing the same type of haptoral ventral bar with five radii, similar to the 'cornu' type[45]. This monophyletic group of *Dactylogyrus* spp. from *Pachychilon* was highly supported by both BI and ML analyses. All *Dactylogyrus* species of *Scardinius* (*D. difformis, D. difformoides* and *D. izjumovae*) formed a highly supported monophyletic group (group C). The group of two *Dactylogyrus* species from *Alburnus* (group B) formed a sister clade to the abovementioned species from *Scardinius*. *Dactylogyrus prostae, D. nanoides*, and *D. folkmanovae* from *Squalius* formed three very strongly supported monophyletic groups (groups D, E, and F, respectively). Group E also clustered with *D. rysavyi* from *A. thessalicus*, *Dactylogyrus* sp. 7 from *C. knerii*, and *Dactylogyrus* sp. 1 from *S. tenellus*, with strong support from both analyses. All three species exhibit a similarly shaped MCO and parasitize phylogenetically closely related cyprinid lineages[26,45].

The phylogenetic relationships between *Dactylogyrus* spp. of *Barbus* and those of *Luciobarbus* were unresolved. However, *Dactylogyrus* spp. of these cyprinids formed three well supported groups (G, H and I). All

No.	Species	LocID	Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	*Alburnoides ohridanus*	A3	MG792846																								
2	*Alburnoides strymonicus*	G2	MG792850	0.008																							
3	*Alburnoides thessalicus*	G3	MG795853	0.003	0.007																						
4	*Chondrostoma nasus*	CZ1	AJ564160	0.013	0.015	0.012																					
5	*Chondrostoma ohridana*	G1	MG792875	0.007	0.011	0.008	0.014																				
6	*Chondrostoma vardarensis*	G3	MG792879	0.014	0.016	0.012	0.010	0.015																			
7	*Chondrostoma phoxinus*	B5	MG792880	0.008	0.012	0.009	0.019	0.013	0.020																		
8	*Leuciscus idus*	CZ	AJ564162	0.011	0.013	0.010	0.002	0.012	0.008	0.017																	
9	*Phoxin ellus alepidotus*	B7	MG792891	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016																
10	*Phoxinellus pseudalepidotus*	B8	MG792892	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016	—															
11	*Squalius cephalus*	CZ1	AJ564161	0.001	0.007	0.002	0.012	0.006	0.013	0.007	0.010	0.006	0.006														
12	*Squalius illyricus*	C3	MG792915	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016	—	—	0.006													
13	*Squalius peloponensis*	G14	MG792918	0.006	0.010	0.007	0.015	0.011	0.018	0.010	0.013	0.009	0.009	0.005	0.009												
14	*Squalius platyceps*	A8	MG792920	0.004	0.008	0.005	0.013	0.009	0.016	0.008	0.011	0.007	0.007	0.003	0.007	0.004											
15	*Squalius prespensis*	A9	KY629340	0.003	0.007	0.004	0.014	0.008	0.015	0.007	0.012	0.006	0.006	0.002	0.006	0.005	0.001										
16	*Squalius prespensis*	G1	MG792925	0.003	0.007	0.004	0.014	0.008	0.015	0.007	0.012	0.006	0.006	0.002	0.006	0.005	0.001	—									
17	*Squalius squalus*	B11	MG792930	0.001	0.007	0.002	0.012	0.006	0.013	0.007	0.010	0.006	0.006	—	0.006	0.005	0.003	0.002	0.002								
18	*Squalius svallize*	C5	MG792932	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016	—	—	0.006	—	0.009	0.007	0.006	0.006	0.006							
19	*Squalius tenellus*	B5	MG792934	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016	—	—	0.006	—	0.009	0.007	0.006	0.006	0.006	—						
20	*Squalius vardarensis*	G4	MG792936	0.001	0.007	0.002	0.012	0.006	0.013	0.007	0.010	0.006	0.006	—	0.006	0.005	0.003	0.002	0.002	—	0.006	0.006					
21	*Telestes fontinalis*	C6	MG792941	0.004	0.008	0.005	0.015	0.009	0.016	0.004	0.013	0.003	0.003	0.003	0.003	0.006	0.004	0.003	0.003	0.003	0.003	0.003	0.003				
22	*Telestes karsticus*	C7	MG792943	0.004	0.008	0.005	0.015	0.009	0.016	0.004	0.013	0.003	0.003	0.003	0.003	0.006	0.004	0.003	0.003	0.003	0.003	0.003	0.003	—			
23	*Telestes metohiensis*	B13	MG792945	0.007	0.011	0.008	0.018	0.012	0.019	0.001	0.016	—	—	0.006	—	0.009	0.007	0.006	0.006	0.006	—	—	0.006	0.003	0.003		
24	*Telestes montenigrinus*	A10	MG792948	0.007	0.010	0.007	0.015	0.004	0.015	0.013	0.013	0.012	0.012	0.006	0.012	0.011	0.009	0.008	0.008	0.006	0.012	0.012	0.006	0.009	0.009	0.012	
25	*Telestes pleurobipunctatus*	G7	MG792949	0.004	0.008	0.005	0.015	0.009	0.016	0.008	0.013	0.007	0.007	0.003	0.007	0.006	0.002	0.001	0.001	0.003	0.007	0.007	0.003	0.004	0.004	0.007	0.009

Table 2. Uncorrected pairwise genetic distances between individuals of *D. vistulae* collected from different host species. Distances are based on partial 18S rDNA combined with ITS1. Identical sequences are marked by dashes (—).

specimens of *D. crivellius*, collected from six *Barbus* species in the Balkans, formed a strongly supported clade. This species clustered with *D. carpathicus* from *B. barbus*. The group of *D. crivellius* and *D. carpathicus* was sister to the group including two *Dactylogyrus* species (sp. 2 and sp. 3) of Balkan *Luciobarbus* spp. (within group I). While *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3 were found to be almost identical on the basis of morphological characters, they differed at the molecular level (concatenated partial 18S rDNA and ITS1 region, p-distance = 0.041). Our results did not support the monophyly of *D. petenyi*, as this species clustered with *D. malleus* and *D. prespensis* (group G). *Dactylogyrus omenti* from *Aulopyge huegelii* appears also to be phylogenetically closely related to the species parasitizing *Barbus* and *Luciobarbus*, but its position was only moderately supported by BI analysis. The position of *D. rosickyi* of *P. pictum* was also uncertain; however, BI analysis strongly supported its position within the clade including groups C–I. *Dactylogyrus rutili* from *Rutilus* formed a well-supported group (group J) and, according to our results, appears to be phylogenetically closely related to *D. suecicus* (whose monophyly was not supported) and *Dactylogyrus* sp. 8 from *T. karsticus*. Surprisingly, *D. ergensi* collected from three host species formed a paraphyletic group. *Dactylogyrus ergensi* from *C. ohridana* was phylogenetically related to

No.	Species	LocID	Accession number	1	2	3	4	5	6	7	8	9	10
1	*Alburnus neretvae*	B1	MG792844										
2	*Alburnus neretvae*	B2	MG792845	0.001									
3	*Pelasgus laconicus*	G11	MG792890	0.025	0.024								
4	*Rutilus basak*	B10	MG792895	0.020	0.019	0.020							
5	*Rutilus lacustris*	B13	MG792899	0.008	0.007	0.017	0.016						
6	*Rutilus ohridanus*	A4	MG792903	0.017	0.016	0.020	0.008	0.016					
7	*Rutilus rutilus*	CZ1	AJ564151	0.009	0.008	0.020	0.017	0.003	0.017				
8	*Telestes alfiensis*	G15	MG792938	0.030	0.029	0.025	0.025	0.022	0.027	0.025			
9	*Telestes dabar*	B12	MG792939	0.021	0.020	0.022	0.018	0.014	0.020	0.014	0.028		
10	*Telestes fontinalis*	C6	MG792940	0.022	0.021	0.024	0.022	0.017	0.020	0.014	0.028	0.010	
11	*Telestes metohiensis*	B13	MG792944	0.023	0.022	0.018	0.020	0.014	0.022	0.017	0.028	0.004	0.012

Table 3. Uncorrected pairwise genetic distances between individuals of *D. rarissimus* collected from different host species. Distances are based on partial 18S rDNA combined with ITS1.

No.	Species	LocID	Accession number	1	2	3	4	5	6	7	8
1	*Squalius cephalus*	B7	MG792911								
2	*Squalius cephalus*	CZ1	MG792912	0.002							
3	*Squalius orpheus*	G9	MG792916	0.018	0.020						
4	*Squalius platyceps*	A8	MG792919	0.016	0.018	0.017					
5	*Squalius prespensis*	A9	MG792921	0.011	0.013	0.013	0.009				
6	*Squalius prespensis*	G1	MG792922	0.010	0.012	0.011	0.007	0.002			
7	*Squalius* sp.	G10	MG792926	0.018	0.020	0.017	0.014	0.013	0.011		
8	*Squalius squalus*	C4	MG792928	0.035	0.037	0.035	0.032	0.028	0.026	0.036	
9	*Squalius vardarensis*	G4	MG792935	0.017	0.019	0.017	0.013	0.010	0.008	0.016	0.032

Table 4. Uncorrected pairwise genetic distances between individuals of *D. folkmanovae* collected from *Squalius* species. Distances are based on partial 18S rDNA combined with ITS1.

D. caucasicus, parasitizing on *Alburnoides* species (group L), in contrast to other *D. ergensi* specimens collected from *C. knerii* and *C. vardarensis*. Nonetheless, *D. caucasicus, D. dirigerus* and *D. ergensi* (included in groups K and L) share a similarly shaped MCO.

The computation of genetic distances between specimens of generalist *Dactylogyrus* species revealed moderate-to-high interpopulation genetic variability. Pairwise genetic distances were calculated for *D. vistulae, D. rarissimus*, and *D. folkmanovae* after eliminating all positions containing gaps and missing data. The selected species are representatives of *Dactylogyrus* with a wide distribution range in Europe. While *D. folkmanovae* is a parasite only of *Squalius* spp., *D. vistulae* and *D. rarissimus* are real generalists parasitizing on species of different cyprinid genera. An alignment of 994 nucleotide positions was used for *D. vistulae* collected from 24 cyprinid species of six genera at 20 localities across the Balkan Peninsula and the Czech Republic. Pairwise sequence diversities varied from 0.000 to 0.020 (Table 2). Generally, geographically adjacent populations were more similar at the molecular level, a finding supported by the Mantel test ($P = 0.015$). *Dactylogyrus vistulae* from *S. tenellus, S. svallize, S. illyricus, Phoxinellus pseudalepidotus, P. alepidotus*, and *T. metohiensis* were genetically identical and all their host species were from the Dalmatian ichthyogeographical district. The same pattern was observed for *D. vistulae* specimens from *C. nasus* and *Leuciscus idus*, both from central Europe: they were similar at the molecular level. One of the few exceptions was *D. vistulae* from *S. cephalus* in the Czech Republic, which was genetically more similar to Balkan populations collected from *S. squalus* and *S. vardarensis* than to central European populations. *Dactylogyrus rarissimus* was collected from 11 species including four cyprinid genera – *Alburnus, Pelasgus, Rutilus* and *Telestes*. After removing gaps and missing data, the final alignment contained a total of 978 nucleotide positions. The interpopulation genetic variability ranged from 0.001 to 0.030 (Table 3). The pairwise distances revealed that *D. rarissimus* from *R. rutilus* and *R. lacustris* were the most similar (p-distance = 0.003). Specimens of *D. rarissimus* from *T. alfiensis* were the most genetically dissimilar to all other specimens collected from other host species (p-distance > 0.021). Regarding *D. rarissimus*, the Mantel test did not reveal any significant spatial genetic structure ($P > 0.05$). *Dactylogyrus folkmanovae* specimens were collected from seven *Squalius* species at nine localities from the Balkans and central Europe. The final alignment contained 977 positions and genetic distances varied from 0.002 to 0.037 (Table 4). Interpopulation genetic variability was found even between specimens collected from two populations of one host species, namely *S. prespensis* (p-distance = 0.002), where both populations were in the same ichthyogeographical district. Surprisingly, the same genetic distance was observed between *D. folkmanovae* specimens collected from *S. cephalus* in Bosnia and Herzegovina and from *S. cephalus* in the Czech Republic. The Mantel test indicated a positive correlation between genetic and geographical distance for *D. folkmanovae* populations ($P = 0.001$).

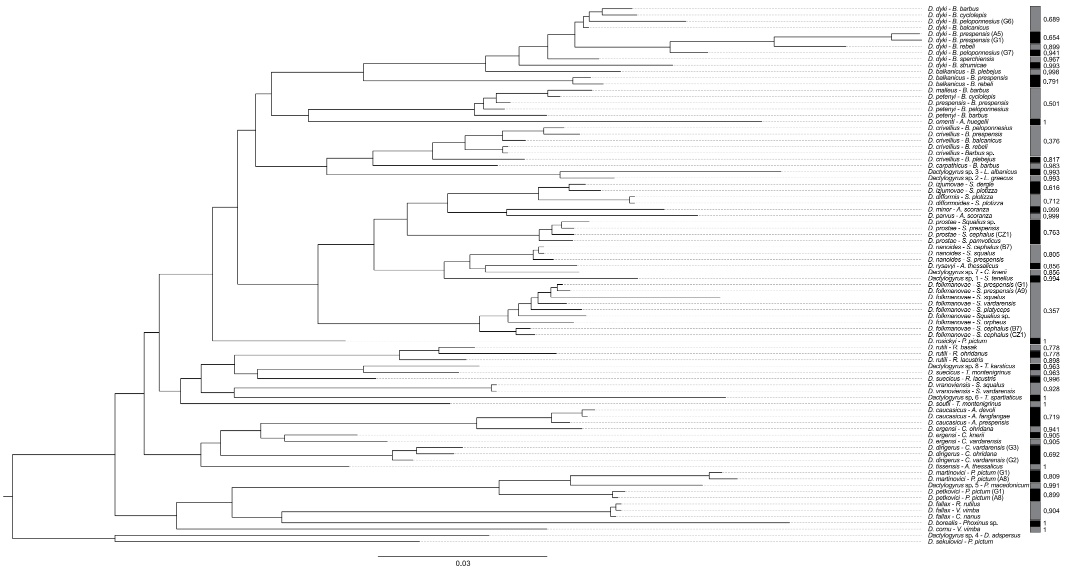

Figure 3. *Results of species PTP delimitation analysis based on the phylogram in* Fig. 2. Vertical bars at terminal branches indicate different species. Values along brackets indicate support values from both maximum likelihood partition and heuristic bayesian search. Species are the same as in Fig. 2 but several branches are rotated.

Species delimitation. The species status of *Dactylogyrus* parasites exhibiting high interpopulation molecular diversity was investigated on the basis of a statistical analysis of our sequence data using PTP. We examined all specimens from clade 2 (Fig. 2). Results of the maximum likelihood analysis (Fig. 3) supported the original species statuses of specimens identified under the following species: *D. dirigerus, D. difformis, D. difformoides, D. izjumovae, D. nanoides, D. prostae, D. folkmanovae*, and *D. vranoviensis*. Specimens of *D. rutili*, collected from three *Rutilus* species, were recognized as three different species. Meanwhile, two molecular variants of *D. suecicus* and the phylogenetically closely related *Dactylogyrus* sp. 8 from *T. karsticus* were also recognized by our analyses as three different species. With respect to *D. dyki*, our analyses suggested six different species. *Dactylogyrus ergensi* specimens from *C. vardarensis, C. knerii*, and *S. squalus* were suggested to be three different species. *Dactylogyrus ergensi* from *C. ohridana* was suggested to be the same species as *D. caucasicus* from *Alburnoides*. Finally, *D. petenyi, D. prespensis* and *D. malleus* were identified as a single species on the basis of clustering methods. The strongest Bayesian supported solution was in congruence with the results of the maximum likelihood solution.

Discussion

The present study suggests that the diversity of *Dactylogyrus* species parasitizing endemic cyprinids in the Balkans is poorer when compared to the diversity of *Dactylogyrus* from central European cyprinids and from cyprinids with a large distribution range (e.g. Šimková *et al.*[11] documented up to 9 different *Dactylogyrus* species from widely distributed *Rutilus rutilus* in the Czech Republic). High numbers of *Dactylogyrus* species were also observed on African cyprinids from the genus *Labeo*, such as *L. coubie* with 9 *Dactylogyrus* species[46]. In contrast, we observed a maximum of 5 *Dactylogyrus* species on a single cyprinid species. These numbers are consistent with previous observations of southern European *Dactylogyrus* fauna, where no more than 5 species were collected from one cyprinid host species[7,44,45]. Such low *Dactylogyrus* species diversity probably has several causes. The distribution range of host species highly influences parasite diversity[47]. Our observations support Gregory's hypothesis[37], i.e. fish species with a wide distribution range are exposed to more parasite species; therefore, they exhibit high parasite diversity. Another potential explanation could be the following: host species with a wide distribution range include a much higher number of populations in comparison to endemic species, which favours parasite speciation. This is illustrated in the present study by *R. rutilus* and *R. aula*. While *R. rutilus*, referred to above as a species with a high *Dactylogyrus* species richness, is the cyprinid species with the widest distribution range in Europe, the distribution area of *R. aula* is limited to the Adriatic basin in Italy and the northwestern Balkans (the Northern Adriatic ichthyogeographical district[15]). *R. aula* is parasitized by a single *Dactylogyrus* species – namely, *D. erhardovae* – in contrast to the aforementioned *R. rutilus*[11]. A similar example concerns the Balkan endemic species *S. illyricus* or *S. peloponensis*, which exhibit very low *Dactylogyrus* species richness (i.e. single species) in comparison to *Squalius cephalus*, from which Seifertová *et al.*[38] documented 9 different *Dactylogyrus* species (up to 14 *Dactylogyrus* species according to the checklist by Moravec[8]). Time of the year when the sampling is performed and the number of investigated populations are known to impact parasite diversity[47,48]. Data on *Dactylogyrus* diversity in cyprinids in central Europe are compiled from numerous studies (i.e. the checklist compiled by Moravec[8]) and include several sampling periods from different river basins, while the present study is focused on a single sampling period in a specific region. The investigated cyprinid hosts endemic to the Balkans are generally distributed in a restricted region where the number of populations potentially harbouring different parasites is expected to be rather lower than in central Europe. Therefore, also following Gregory's hypothesis, we expected lower parasite diversity in endemic cyprinids with a restricted distribution range. Only a few host species, such as *S. squalus*, were collected from several distinct localities; however, the different host populations did not differ in their numbers of *Dactylogyrus* species. It was also shown that the composition of monogenean communities is influenced by environmental factors, especially water temperature. In such cases, shifts in the species compositions of monogenean communities within host species were observed throughout the year[49–53].

The present phylogenetic analyses revealed four well-to-moderately supported clades including both endemic and non-endemic *Dactylogyrus* species, while four species – namely, *D. erhardovae, D. crucifer, D. caballeroi*, and *D. rarissimus* (all parasites of *Rutilus* spp.) – had external positions to these clades. *Dactylogyrus erhardovae* is considered to be a genus specific parasite of *Rutilus*, the first description of this species originating from *R. rubilio*[54], an endemic species of the Apennine Peninsula[55,56]. In the Balkans, *Dactylogyrus erhardovae* was also found on *R. aula* and *R. basak*, phylogenetically closely related species[26,57] distributed in the rivers of the Adriatic Sea basin, which is the proximal ichthyogeographic district to the Tyrrhenian Sea basin, where *R. rubilio* occurs. *Dactylogyrus crucifer* was originally described from *Rutilus rutilus*, but Šimková *et al.*[12] collected this species also from *Leuciscus idus* and *Scardinius erythrophthalmus* and therefore suggested that *D. crucifer* represents a generalist species. In our study, *D. crucifer* was only collected from *Rutilus* species (*R. rutilus* from the Czech Republic and *R. lacustris* from the Ponto-Caspian area), which supports the association between *Rutilus* hosts and *D. crucifer* and even indicates that the occurrence of this parasite on other cyprinid species may be the result of accidental infection. Both *Rutilus* species parasitized by *D. crucifer* originated and live in sympatry in the Black Sea and Caspian Sea basins[58], which may promote the host switching of *D. crucifer* between these two sister *Rutilus* lineages.

Interestingly, we showed that *Dactylogyrus* sp. 4 from *D. adspersus* and *D. sekulovici* from *P. pictum* clustered together (group 1). Both *Dactylogyrus* species seem to be host specific - at least, there are no previous records of these two species from other cyprinid species. Regarding the morphology of the hard parts, these two *Dactylogyrus* species differ in the shape of their MCOs. While *Dactylogyrus* sp. 4 has hard parts morphologically similar to those of *D. erhardovae* from *Rutilus*, it shares with *D. sekulovici* only the shape of the haptoral connective bars (see Pugachev *et al.*[45] for morphology of *D. sekulovici*). Two cyprinid species – namely, *D. adspersus* and *P. pictum* – are representatives of two phylogenetically unrelated ancient lineages[26], but have a similar geographical distribution, i.e. they are restricted to the rivers of the Adriatic Sea Basin. *Pachychilon pictum* occurs only in the Albanian ichthyogeographical district[59]; *D. adspersus* inhabits the central Adriatic (Dalmatian) district, which shares only two species with the Danubian basin[59–61], and is probably linked to the Adriatic district by underground connections[16]. The paraphyly of the *Dactylogyrus* species from *P. pictum* suggests their multiple origin on this host. The phylogenetic proximity of *D. sekulovici* to *Dactylogyrus* sp. 4 suggests a host switch between two cyprinid species living in the same area of the central Adriatic region. The second host-specific parasite of *P. pictum* is *D. ivanovichi*[44,45]. Its phylogenetic position suggests a different origin (when compared to *D. sekulovici*), likely also resulting from a host switch. *Dactylogyrus ivanovichi* is phylogenetically closely related to *D. auriculatus* from *Abramis brama*. The two species exhibit MCOs with an identical structure and differ only in the positioning of the VA and in the root lengths of haptoral anchor hooks[45]. These two species, like the two species of the sister clade (clade 3), secondarily lost their connective haptoral ventral bar[45]. The phylogenetic proximity of *D. ivanovichi* and *D. auriculatus* and the morphological similarities in copulatory organs between *D. ivanovichi* and *Dactylogyrus* spp. of *A. brama* suggest that *D. ivanovichi* originated from a recent host switch from the widely distributed *A. brama*, and then adapted its attachment organ to new host species. Other *Dactylogyrus* species from *P. pictum*, namely *D. martinovici* and *D. petkovici*, are phylogenetically closely related to *Dactylogyrus* sp. 5 of *P. macedonicum*. *Dactylogyrus martinovici*, *D. petkovici*, and *Dactylogyrus* sp. 5 exhibit haptoral hard parts with an almost identical shape but differ in the shapes of their copulatory organs. This is in congruence with Šimková *et al.*[6], suggesting similar adaptations of the haptor among *Dactylogyrus* species parasitizing phylogenetically related hosts. We can hypothesize that these three species evolving from the same ancestor have for a long time been associated with *Pachychilon* and that *D. martinovici* and *D. petkovici* emerged as a result of more recent intra-host duplication followed by reproductive isolation. In contrast, *D. ivanovichi* and *D. sekulovici* are the result of earlier host switching between cyprinid species of different genera living in contact zones and of subsequent speciation. Finally, another *Dactylogyrus* species from *P. pictum*, *D. rosickyi*, exhibits a different phylogenetic position when compared to the aforementioned *Dactylogyrus* of *Pachychilon* spp., which suggests a different origin for this species.

Regarding *Dactylogyrus* from *Barbus* spp., our analyses did not fully resolve the phylogenetic relationships between these species, but in general all species are clustered in three well or moderately supported groups (G–I). In total, we collected 5 different *Dactylogyrus* species from 10 *Barbus* hosts. The most common was *D. dyki*, parasitizing 8 *Barbus* species and representing one clade in our phylogenetic analysis. Šimková *et al.*[43] observed significant interpopulational phenotypic plasticity and molecular variability among *D. dyki* isolated from 3 *Barbus* species, which is in accordance with the present study. The monophyly of the group including *D. dyki* specimens was supported. However, low support for *D. dyki* from *B. strumicae* was found and these specimens were recognized as a different species by species delimitation analysis. Following the suggestion of Šimková *et al.*[43], *D. dyki* from *Barbus* spp. could represent a species complex of several morphologically similar species. The confirmation of this hypothesis requires further morphological reevaluation of *Dactylogyrus* representatives from all *Barbus* hosts, including those from *B. meridionalis* in Western Europe and *B. tyberinus* from the Apennines. We inferred some paraphyly concerning *D. balkanicus*. Whilst *Dactylogyrus* specimens of *B. prespensis* and *B. rebeli* were clustered together, specimens from *B. plebejus* appeared to be phylogenetically related to *D. dyki*. The sister status of these two species is supported by the similar shape of the sclerotized parts of their haptors (both species share a small triangular connective ventral bar), and also the remarkably similar shape of their MCOs[45]. Both species were collected from *B. rebeli* and *B. prespensis*, phylogenetically closely related *Barbus* species[25,62], suggesting (1) historical intra-host speciation, i.e. parasite duplication on their common ancestor and a later host switch

Host	LocID	NH	N	Locality	Main river basin	Coordinates
Abramis brama	CZ1	5	2	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E
Alburnoides devolli	A1	6	1	Devoli, Maliq	Seman	40°42′57.07″N 20°40′54.06″E
Alburnoides fangfangae	A2	7	1	Osum, Vodice	Seman	40°24′13.07″N 20°39′04.04″E
Alburnoides ohridanus	A3	10	1	Fani i Vogel, Reps	Seman	41°52′51.01″N 20°04′44.04″E
Alburnoides prespensis	G1	5	1	Aoos, Kalithea	Aoos	40°01′16.67″N 20°41′40.19″E
Alburnoides strymonicus	G2	5	2	Angistis, between Alistrati & Drama	Strymon	41°05′42.08″N 24°00′18.29″E
Alburnoides thessalicus	G3	12	3	Pinios, Rongia - Valamandrio	Pinios	39°33′07.85″N 21°42′08.02″E
Alburnus neretvae	B1	7	2	Mušnica, Avtovac	Neretva	43°08′42.05″N 18°35′45.00″E
	B2	10	2	Zagorje, Jabuke	Neretva	43°32′18.53″N 17°12′34.28″E
Alburnus scoranza	A4	5	2	Skadar lake, Shiroke	Ohrid-Drin-Skadar lake system	42°03′24.94″N 19°28′07.05″E
Aulopyge hugelii	B3	14	2	Šujica, Duvansko Polje	Neretva	43°42′05.07″N 17°15′50.05″E
Barbus balcanicus	G4	5	3	Gallikos, Mandres	Gallikos	40°59′28.35″N 22°33′14.49″E
Barbus barbus	CZ1	5	3	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E
Barbus cyclolepis	G5	3	2	Macropotamos River	Filiouri	41°04′13.00″N 25°32′52.00″E
Barbus peloponnesius	G6	8	1	Neda, Gianitsochori	Neda	37°23′04.34″N 21°41′24.15″E
	G7	5	3	Kokitos, Pagrati	Acheron	39°26′53.02″N 20°30′03.06″E
Barbus plebejus	C1	7	2	Bribirske Mostine, Bribišnica	Krka	43°55′28.21″N 15°48′45.07″E
Barbus prespensis	A5	5	1	Shkumbini, Perrenjas	Shkumbini	41°03′50.09″N 20°33′56.06″E
	G1	5	4	Aoos, Kalithea	Aoos	40°01′16.67″N 20°41′40.19″E
Barbus rebeli	A6	7	3	Mat, Klos	Mat	41°29′37.01″N 20°05′29.04″E
Barbus sp.	A7	6	1	Kiri	Ohrid-Drin-Skadar lake system	42°08′56.02″N 19°39′42.01″E
Barbus sperchiensis	G8	4	1	Sperchios, Ypati	Sperchios	38°54′14.33″N 22°17′30.22″E
Barbus strumicae	G9	5	1	Rihios river, Stavros	Volvi lake	40°40′16.34″N 23°39′50.87″E
Carassius gibelio	CZ2	5	1	Dyje River	Danube	48°48′09.04″N 16°50′19.03″E
	C2	10	2	Baštica reservoir	Baštica	44°11′42.37″N 15°24′32.13″E
Chondrostoma knerii	B4	5	2	Rečina river, near Jelim lake, Hutovo Blato	Neretva	43°03′39.72″N 17°48′29.30″E
Chondrostoma nasus	CZ1	5	1	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E
Chondrostoma ohridana	G1	4	3	Aoos, Kalithea	Aoos	40°01′16.67″N 20°41′40.19″E
Chondrostoma phoxinus	B5	11	1	Šujica, Šujicko Polje	Neretva	43°49′41.43″N 17°10′48.20″E
Chondrostoma vardarensis	G2	3	1	Angistis river, Koninogia	Strymon	41°11′36.41″N 23°54′25.00″E
	G2	2	1	Angistis, between Alistrati & Drama	Strymon	41°05′42.08″N 24°00′18.29″E
	G3	1	2	Pinios, Rongia - Valamandrio	Pinios	39°33′07.85″N 21°42′08.02″E
Delminichthys adspersus	B6	6	1	Nezdravica, Tihaljina	Neretva	43°19′00.05″N 17°23′20.01″E
Luciobarbus albanicus	G10	4	1	Trichonis lake, Panetolio	Acheloos	38°35′20.19″N 21°28′02.68″E
Luciobarbus graecus	G7	10	1	Sperchios, Ypati	Sperchios	38°54′14.33″N 22°17′30.22″E
Pachychilon macedonicum	G3	8	1	Pinios, Rongia - Valamandrio	Pinios	39°33′07.85″N 21°42′08.02″E
Pachychilon pictum	A8	4	2	Ohrid lake	Ohrid-Drin-Skadar lake system	41°04′27.08″N 20°37′40.00″E
	G1	5	5	Aoos, Kalithea	Aoos	40°01′16.67″N 20°41′40.19″E
Pelasgus laconicus	G11	13	1	Evrotas, Sparti	Evrotas	37°05′34.70″N 22°25′34.81″E
Phoxinellus alepidotus	B7	12	1	Bosansko Grahovo, Korana river	Korana	44°10′37.00″N 16°23′03.61″E
Phoxinellus pseudalepidotus	B8	10	1	Lištica, Polog	Neretva	43°20′32.09″N 17°41′37.04″E
Phoxinus sp.	B9	14	1	Zalomka, Ribari	Neretva	43°15′26.04″N 18°21′41.05″E
Rutilus aula	C2	10	1	Baštica river, Grabovač reservoir	Baštica	44°11′42.37″N 15°24′32.13″E
Rutilus basak	B10	13	4	Krenica lake, Drinovci	Neretva	43°22′25.00″N 17°19′59.04″E
Rutilus lacustris	G12	3	4	flood pools by Struma, Lithopos	Strymon	41°07′40.41″N 23°16′24.70″E
Rutilus ohridanus	A4	4	4	Skadar lake, Shiroke	Ohrid-Drin-Skadar lake system	42°03′24.94″N 19°28′07.05″E
Rutilus rutilus	CZ1	5	3	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E
Scardinius dergle	C1	10	1	Bribirske Mostine, Bribišnica	Krka	43°55′28.21″N 15°48′45.07″E
Scardinius plotizza	B4	7	3	Rečina river, near Jelim lake, Hutovo Blato	Neretva	43°03′39.72″N 17°48′29.30″E
Squalius cephalus	CZ1	5	2	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E
	B7	4	2	Bosansko Grahovo, Korana river	Korana	44°10′37.00″N 16°23′03.61″E
Squalius illyricus	C3	8	1	Cetina river, Kosore	Cetina	43°56′29.78″N 16°26′23.37″E
Squalius orpheus	G9	4	1	Rihios river, Stavros	Volvi lake	40°40′16.34″N 23°39′50.87″E
Squalius pamvoticus	G13	6	1	Acheron, Gliki	Acheron	39°19′00.05″N 20°36′04.03″E

Continued

Host	LocID	NH	N	Locality	Main river basin	Coordinates
Squalius peloponensis	G14	5	1	Pamissos, Vasiliko	Pamissos	37°15′17.39″N 21°53′45.15″E
Squalius platyceps	A8	5	2	Ohrid lake	Ohrid-Drin-Skadar lake system	40°59′00.66″N 20°38′23.40″E
Squalius prespensis	A9	4	2	Shkumbini, Pajove	Shkumbini	41°03′31.07″N 19°51′47.03″E
	G1	6	3	Aoos, Kalithea	Aoos	40°01′16.67″N 20°41′40.19″E
Squalius sp.	G10	2	2	Trichonis lake, Panetolio	Acheloos	38°35′20.19″N 21°28′02.68″E
Squalius squalus	B11	10	3	Donja Drežnica, Drežnica river	Drežnica	43°31′31.46″N 17°42′51.66″E
	C4	11	1	Pazin, Pazinčica river	Pazinčica	45°14′47.92″N 13°58′10.66″E
Squalius svallize	C5	15	1	Konavočica, Grude	Ljuta	42°31′33.86″N 18°22′04.16″E
Squalius tenellus	B5	11	2	Šujica, Šujičko Polje	Neretva	43°49′41.43″N 17°10′48.20″E
Squalius vardarensis	G4	4	3	Gallikos, Mandres	Gallikos	40°52′07.33″N 22°53′59.12″E
Telestes alfiensis	G15	5	1	Erimantos, Tripotamo	Alfios	37°52′37.07″N 21°53′15.05″E
Telestes dabar	B12	3	1	Vrijeka, Dabarsko Polje	Neretva	43°03′32.07″N 18°14′39.04″E
Telestes fontinalis	C6	13	2	Krbavsko polje, Laudonov gaj	Krbava	44°38′14.33″N 15°40′05.65″E
Telestes karsticus	C7	10	2	Drežnica, Sušik river	Drežnica	45°08′44.13″N 15°04′41.56″E
Telestes metohiensis	B13	5	2	Zalomka, Nevesinjsko polje	Neretva	43°12′06.06″N 18°12′21.07″E
Telestes montenigrinus	A10	10	3	Skadar lake, Shegan	Ohrid-Drin-Skadar lake system	42°16′22.09″N 19°23′39.09″E
Telestes pleurobipunctatus	G7	6	1	Kokitos, Pagrati	Acheron	39°26′53.02″N 20°30′03.06″E
Tropidophoxinellus spartiaticus	G6	5	1	Neda, Gianitsochori	Neda	37°23′04.34″N 21°41′24.15″E
Vimba vimba	CZ1	5	3	Svratka River	Danube	49°05′32.01″N 16°37′11.00″E

Table 5. List of cyprinid species including the localities of their collection. LocID = codes used in all tables and figures, NH = number of host specimens processed, N = number of *Dactylogyrus* species collected.

to another endemic *Barbus*, or (2) parasite duplication on recent *Barbus* species in this region and a host switch to the phylogenetically and geographically closest *Barbus* species. According to our phylogenetic analyses, *D. petenyi*, *D. malleus*, and *D. prespensis* form a well-supported group, namely group G. These three *Dactylogyrus* species parasitizing *Barbus* species share similar morphologies of the copulatory organs and haptoral hard parts. Surprisingly, specimens of *D. petenyi* do not form a monophyletic group. Species delimitation analysis suggests that each representative of group G represents a single species.

Specimens of *D. crivellius* from different host species formed a monophyletic group. Our phylogenetic analyses support a monophyletic group including *D. crivellius* from Balkan *Barbus* spp., *D. carpathicus* from *B. barbus*, and *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3. These four species exhibit the same morphology of a ventral bar with 5 extremities, a typical feature of *Dactylogyrus* spp. from *Luciobarbus*. Species with this morphology are considered as the 'carpathicus'[42] or 'cornu'[45] type. This supports the hypothesis that haptoral hard parts are more suitable for resolving the phylogeny of monogeneans; that is, haptor morphology is similar between closely related species[6,63,64].

The phylogenetic position of *D. omenti* among *Dactylogyrus* species parasitizing *Barbus* and *Luciobarbus* was already suggested by Benovics *et al.*[65]. Even though its exact phylogenetic position is not fully resolved, our result suggests that this species is phylogenetically closer to *D. petenyi* and *D. prespensis* than to the aforementioned species which share the 'cornu' type of haptoral ventral bar. Adding more *Dactylogyrus* species from Iberian, North African, and Middle Eastern *Barbus* and *Luciobarbus* in a phylogenetic reconstruction and assessing coevolutionary scenarios involving these parasites and their hosts could better resolve the relationships within this group of *Dactylogyrus*.

Several well-supported phylogenetic groups (J–L) were formed exclusively by *Dactylogyrus* species of the 'ergensi' type of copulatory organ, or, in the case of *D. tissensis*, the 'chondrostomi' type of copulatory organ[47]. While the MCO and VA among *Dactylogyrus* spp. belonging to groups J–L are very similar, these species differ in the shapes and sizes of their haptoral hard parts. All *Dactylogyrus* species of groups K and L parasitize species of the genera *Alburnoides* and *Chondrostoma*. The species status of *D. caucasicus* parasitizing *Alburnoides* and that of *D. dirigerus* parasitizing *Chondrostoma* were supported by species delimitation analysis. Surprisingly, *Rutilus*-specific *D. rutili* belonging to the phylogenetically distant group J possesses the same type of copulatory organ as *D. caucasicus* and *D. dirigerus*. This suggests that a similar copulatory organ morphotype can emerge independently several times during the evolution of *Dactylogyrus* species in evolutionarily distant hosts (such are *Rutilus, Chondrostoma*, and *Alburnoides*[26]). Rohde[2] hypothesized that the rapid evolution of morphological variation in copulatory organs is considered as a mechanism for avoiding hybridization. In contrast, similar types of copulatory organs in *Dactylogyrus* species may be recognized in different host lineages, as shown in the present study. Then, species with a similar MCO morphotype could be found within congeneric hosts only if these parasite lineages had diversified recently (e.g. *D. ergensi* and *D. dirigerus* of *Chondrostoma*).

High numbers of southern European endemic *Dactylogyrus* species were strictly host specific and/or distributed only in one region. However, some of them were collected from a wide range of cyprinid hosts. *Dactylogyrus vistulae* is the species with the widest host range in the Balkans. In addition to the host range for this parasite revealed in this study, the presence of *D. vistulae* was also reported from *R. rutilus* in Finland[66] and from *V. vimba* in the Czech Republic[8]. Genetic distances between specimens collected from different host species correlated with

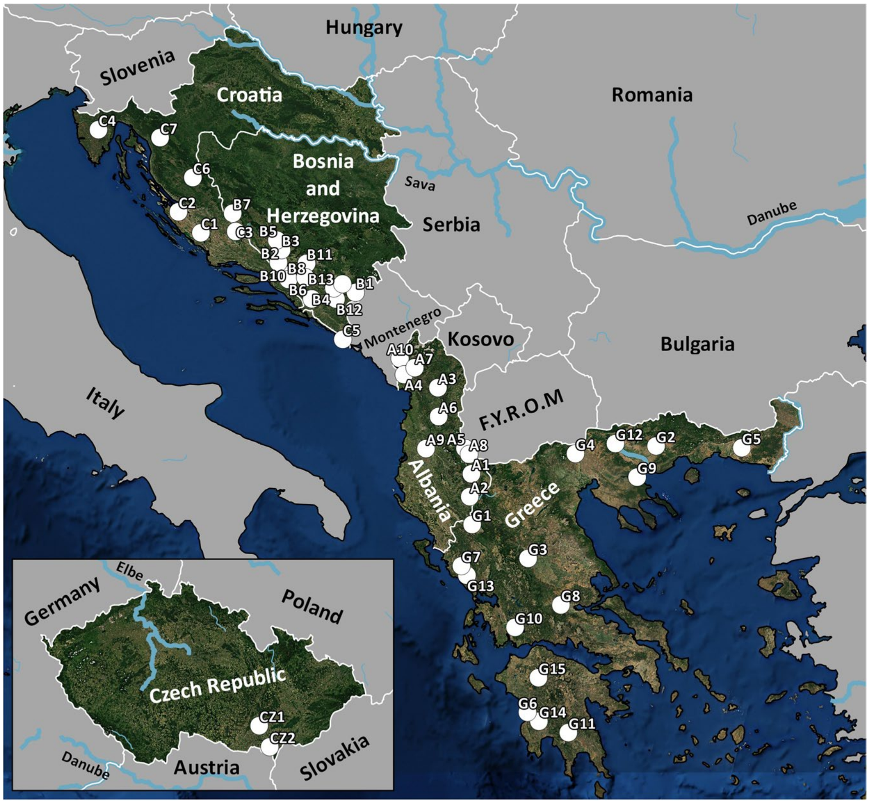

Figure 4. *Map of collection localities in the Balkans*. The sames codes for localities are used in tables under the label LocID. The map was generated in QGIS 3.0.3[94].

geographical distances, suggesting the geographical structure of *D. vistulae* populations, rather than some association with the phylogenetic relatedness of the host species. For example, *D. vistulae* from *C. phoxinus* appears to be genetically more similar to *D. vistulae* from hosts in the same or close ichthyogeographical region than to *D. vistulae* collected from geographically separated congeneric *Chondrostoma*. Since *D. vistulae* is widely distributed and relatively easily distinguishable from other *Dactylogyrus* spp. on the same hosts (on the basis of morphological characters and its large body size[45]), it could potentially represent a suitable model for population studies that could elucidate the origin of this species and the distribution pattern between phylogenetically distant hosts or between two host species from different regions. Another species with a wide distribution range is *D. rarissimus*. It was originally considered as a specialist of *R. rutilus*[6,12,67]; however, we collected this species in the Balkans from phylogenetically well-separated genera: *Rutilus*, *Alburnus, Pelasgus* and *Telestes*. In this case, the Mantel test did not reveal a significant correlation between genetic and geographical distances, even as specimens collected from *T. alfiensis* and *P. laconicus* in Peloponnese (the only representatives of *D. rarissimus* from the Ionian ichthyogeographic district) are genetically the most different from northern populations originating from the Albanian district (such as *R. ohridanus*). We measured only a very small genetic difference between *D. rarissimus* from *R. rutilus* and *D. rarissimus* from *R. lacustris* (similarly to that measured for *D. crucifer*), which supports the recent divergence of these *Rutilus* species or, alternatively, a more ancient separation followed by recent contact. All these results suggest that *D. rarissimus* is a true generalist species parasitizing several cyprinid genera. We investigated the correlation between genetic and geographical distances among *D. folkmanovae* individuals. In contrast to *D. vistulae* and *D. rarissimus*, *D. folkmanovae* was reported as a generalist parasite of *S. cephalus* and *R. rutilus*[8,67]; however, it is generally reported in *Squalius* species[12] and, in the Balkans, *D. folkmanovae* occurs strictly on *Squalius* spp. *Dactylogyrus folkmanovae* from *S. squalus* appeared to be the most genetically different from individuals parasitizing other host species. Of the southern European endemic *Squalius* species, *Squalius squalus* exhibits the largest distribution range, i.e. it covers the whole peri-Adriatic region[15], and is phylogenetically closely related to *S. prespensis*[26]. This is in congruence with measurements of genetic distance, according to which *D. folkmanovae* of *S. squalus* and *S. prespensis* are the most similar. These results suggest that *D. folkmanovae* of *S. squalus* is the oldest lineage within this species in the Balkans. In contrast, representatives of *D. folkmanovae* from *S. cephalus* in the Czech Republic and *D. folkmanovae* from *S. cephalus* in Bosnia and Herzegovina are genetically very similar. These small genetic distances (in the case of both *D. vistulae* and *D. folkmanovae*) could be the result of more recent contact between hosts from these two distant regions via underground connections, as proposed by Palandačić *et al.*[16], or through the introduction of non-native species/populations into the Balkan region. Fish introduction has been a very common occurence in the Balkans and includes both exotic, and native species from geographically near localities[68,69]. River drainages[70,71] and also isolated karstic drainages are affected, where non-native species such as *S. cephalus* and *R. rutilus* have been introduced[72]. Low molecular variability between

Czech and Bosnian-Herzegovinian populations of *D. folkmanovae* may favour the hypothesis of the natural dispersion of the fish via river connections. However, the investigation of other European populations and the use of other genetic markers suitable for population genetics of *Dactylogyrus* are necessary to reveal the distribution patterns of widespread *Dactylogyrus* species. In addition, the extent of parasite transfer from introduced species to endemic species needs to be studied further to reduce the possible risk of parasite introduction to already threatened native species.

In this study, we revealed interpopulation genetic variability within endemic Balkan *Dactylogyrus* species. The intraspecific genetic distances could also be linked to the morphological variability which was suggested for other monogenean taxa[73–75]. Concerning *Dactylogyrus*, morphological variability among the haptoral hard parts of a given *Dactylogyrus* species was recorded even within a single host specimen of *L. maghrebensis*[71], but without any molecular variability, suggesting phenotypic plasticity and/or selection within a specific microhabitat. On the other hand, as documented above, our molecular data also revealed potential complexes of cryptic species, formerly considered to be a single species solely on the basis of a morphological approach. According to species delimitation analysis, the 38 *Dactylogyrus* species included in the analysis may in fact represent 47 species. This finding is in accordance with previous studies, in which delimitation analyses were incongruent with classical taxonomy[76,77]. In our study, *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3 from *L. graecus* and *L. albanicus*, respectively, were shown to be morphologically indistinguishable species; however, molecular data suggest that they are actually two different species (which is also supported by species delimitation analysis). A similar result was revealed for other *Dactylogyrus* species, such as *D. rutili*, which seems, on the basis of delimitation analysis, to represent three species parasitizing three host species, and *D. dyki*, which seems to represent six potential species on 10 *Barbus* host species. Our future aim will be to undertake the morphometrical reevaluation of taxonomically important traits in combination with the use of molecular data in order to resolve the potential species complexes previously recognized within *Dactylogyrus*[76].

Material and Methods

Parasite sampling. From 2014 to 2017, individuals from 63 cyprinid fish species were sampled from 47 different localities in the Balkan Peninsula and the Czech Republic (Table 5, Fig. 4). Approximately 90% of all endemic cyprinid species in the Balkans were processed in this study[15]. Fish were dissected using the standard methods described by Ergens and Lom[78] and their *Dactylogyrus* species were collected. More precisely, *Dactylogyrus* specimens were removed from the gills, mounted on slides, and covered in a mixture of glycerine and ammonium picrate (GAP[79]) for further determination. All applicable institutional, national and international guidelines for the care and use of animals were followed and approved by the Animal Care and Use Committee of the Faculty of Science, Masaryk University in Brno (Czech Republic). Identification at the species level was performed using an Olympus BX51 microscope equipped with phase contrast optics. *Dactylogyrus* species were determined using Pugachev *et al.*[45] on the basis of the size and shape of the hard parts of the attachment organ (the haptor) and the reproductive organs (MCO and VA). Some *Dactylogyrus* specimens from each cyprinid species investigated were bisected using fine needles under a dissecting microscope, and the body part with the haptor was individually preserved in 96% ethanol for further DNA extraction. The remaining body part, i.e. that including the hard parts of the respective reproductive organ, was mounted on a slide for species determination.

DNA extraction, amplification, and sequencing. Individual parasites were removed from the ethanol and dried using a vacuum centrifuge. DNA was extracted using the standard protocol (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Partial 18S rDNA and the the entire ITS1 region were amplified using the primers S1 (5′-ATTCCGATAACGAACGAGACT-3′) and IR8 (5′-GCTAGCTGCGTTCTTCATCGA-3′)[80], which anneal to the 18S and 5.8S rDNA respectively. Partial 28S rDNA was amplified using the following primers: forward C1 (5′-ACCCGCTGAATTTAAGCA-3′) and reverse D2 (5′-TGGTCCGTGTTTCAAGAC-3′)[81]. Each amplification reaction for partial 18S rDNA and the ITS1 region was performed in a final volume of 15 µl, containing 1.5 units of Taq polymerase, 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 2.5 µl of DNA (20 ng/µl). PCR was carried out using the following steps: 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1 min 30s at 72 °C, and 10 minutes of final elongation at 72°C. The PCR for partial 28S was performed using the same conditions as described in Šimková *et al.*[82]. The PCR products were checked on 1% agarose gel and purified using ExoSAP-IT kit (Ecoli, Bratislava, SK) following the standard protocol. Purified products were directly sequenced using the PCR primers and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems). New sequences were deposited in GenBank (their accession numbers are shown with asterisks in Table 5).

Phylogenetic analyses. DNA sequences were aligned using fast Fourier transform in MAFFT[83]. The sequences were trimmed to concur with *Dactylogyrus* sequences obtained from GenBank. The sequences for 14 *Dactylogyrus* species from central European cyprinids were obtained by sequencing in this study or acquired from GenBank (see Table 5 for accession numbers).

Genetic distances between specimens of selected *Dactylogyrus* species collected from different host species were computed using sequences of partial 18S rDNA combined with ITS1 region. Uncorrected pairwise distances were calculated in MEGA 7[84].

Gaps and ambiguously aligned regions were removed from the alignment using GBlocks v. 0.91[85]. Phylogenetic analyses using maximum likelihood were computed with RaxML v8.1.X[86], and by means of Bayesian inference with MrBayes 3.2[87]. For each analysis, jModelTest 2.1.10 was employed to select the most appropriate model of DNA evolution[88,89] using the Bayesian information criterion (BIC). Trees obtained by ML analyses were validated using 1000 bootstrap iterations. Bayesian inference was performed using the Metropolis-coupled Markov chain

Monte Carlo algorithm, with 2 parallel runs of 1 cold and 3 hot chains. This was run for 10^7 generations and trees were sampled every 10^2 generations. 30% of all saved trees were discarded as a relative burn-in period according to the standard deviation split frequency value (<0.01).

Phylogenetic reconstruction including all sampled *Dactylogyrus* species was based on concatenated sequences of partial 18S rDNA and partial 28S rDNA (Fig. 1). The resulting phylogram was rooted using the evolutionarily divergent lineage of *Dactylogyrus* species parasitising *Carassius gibelio* and *Cyprinus carpio*[12]. To resolve the phylogenetic relationships among specific subgroups, partial subtree analyses were performed using partial 18S rDNA combined with the ITS1 region and partial 28S rDNA. Optimal evolutionary models were selected for each marker using BIC, each model including an alpha parameter for the gamma distribution (G) accounting for rate heterogeneity across sites and/or a proportion of invariable sites (I).

Species delineation in the final trees was carried out using a PTP (Poisson Tree Processes) model[90]. This approach was applied to the BI tree computed from concatenated partial 18S rDNA, 28S rDNA, and the partial ITS1 region, and run for 5×10^5 generations. 30% of the resulting trees were discarded as burn-in. PTP can give species delimitation hypothesis based on gene trees inferred from molecular sequences, modelling the speciation or branching events in terms of the number of mutations. This method does not require an ultrametric input tree or a sequence similarity threshold as input, but uses only the tree resulting from either phylogenetic reconstruction.

The Mantel test[91] to test the correlation between genetic and geographical distances was performed in R[92] using the *mantel* function in the *vegan* package[93].

Data Availability

All new sequences of Dactylogyrus obtained during this study were deposited in NCBI GenBank under accession numbers MG792838–MG793066. Appropriate accession numbers according to Dactylogyrus species and specific rDNA regions are presented in Tables 1–3. Since whole fish specimens were completely processed during parasitological dissection, additional specimens of each analysed host species were collected from the same locality and fish vouchers were deposited in the ichthyological collection of the National Museum in Prague (Czech Republic). Voucher specimens of the sequenced Dactylogyrus species (excluding undescribed species) are deposited in the Finnish Museum of Natural History in Helsinki (available under the accession numbers MZH KN10850–989).

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Author Contributions

A.Š. designed and supervised the study. A.Š., J.V. and R.Š. organized the field trip and fish and parasite collection. J.V. and R.Š. collected and identified fish in the field and provided the background on the host phylogeny and distribution. A.Š. and M.B. processed fish and collected parasites during the field trip. M.B. performed microscopical observations, determination of *Dactylogyrus* species and all laboratorty procedures. M.B. and Y.D. performed phylogenetic and statistical analyses. M.B. wrote the draft of the manuscript. A.Š., Y.D., J.V. and R.Š. revised the manuscript. All authors read and approved final version of manuscript.

Additional Information

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PAPER IV

Cophylogenetic relationships between *Dactylogyrus* (Monogenea) ectoparasites and endemic cyprinoids of the north-eastern European peri-Mediterranean region

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Cophylogenetic relationships between *Dactylogyrus* (Monogenea) ectoparasites and endemic cyprinoids of the north-eastern European peri-Mediterranean region

Michal Benovics[1] | Yves Desdevises[2] | Radek Šanda[3] | Jasna Vukić[4] | Andrea Šimková[1]

[1]Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic

[2]CNRS, Biologie Intégrative des Organismes Marins (BIOM), Observatoire Océanologique, Sorbonne Université, Banyuls/Mer, France

[3]National Museum, Prague, Czech Republic

[4]Department of Ecology, Faculty of Science, Charles University, Prague, Czech Republic

Correspondence
Michal Benovics, Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, Brno 61137, Czech Republic.
Email: benovics@mail.muni.cz

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Abstract

The study of host–parasite coevolution is one of the cornerstones of evolutionary biology. The majority of fish ectoparasites belonging to the genus *Dactylogyrus* (Monogenea) exhibit a high degree of host specificity. Therefore, it is expected that their evolutionary history is primarily linked with the evolutionary history of their cyprinoid fish hosts and the historical formation of the landmasses. In the present study, we used a cophylogenetic approach to investigate coevolutionary relationships between endemic Cyprinoidea (Cyprinidae and Leuciscidae) from selected regions in southern Europe and their respective *Dactylogyrus* species. A total of 49 *Dactylogyrus* species including endemic and non-endemic species were collected from 62 endemic cyprinoid species in the Balkan and Apennine Peninsulas. However, 21 morphologically identified *Dactylogyrus* species exhibited different genetic variants (ranging from 2 to 28 variants per species) and some of them were recognized as cryptic species on the basis of phylogenetic reconstruction. Phylogenetic analyses revealed several lineages of endemic and non-endemic *Dactylogyrus* species reflecting some morphological similarities or host affinities. Using distance-based and event-based cophylogenetic methods, we found a significant coevolutionary signal between the phylogenies of parasites and their hosts. In particular, statistically significant links were revealed between *Dactylogyrus* species of Barbini (Cyprinidae) and their hosts belonging to the genera *Aulopyge*, *Barbus* and *Luciobarbus*. Additionally, a strong coevolutionary link was found between the generalist parasites *D. alatus*, *D. sphyrna*, *D. vistulae*, and their hosts, and between *Dactylogyrus* species of *Pachychilon* (Leuciscidae) and their hosts. Cophylogenetic analyses suggest that host switching played an important role in the evolutionary history of *Dactylogyrus* parasitizing endemic cyprinoids in southern Europe. We propose that the high diversification of phylogenetically related cyprinoid species in the Mediterranean area is a process facilitating the host switching of specific parasites among highly diverse congeneric cyprinoids.

Contributing authors: Yves Desdevises (desdevises@obs-banyuls.fr); Radek Šanda (radek_sanda@nm.cz); Jasna Vukić (jasna.vukicova@natur.cuni.cz); Andrea Šimková (simkova@sci.muni.cz)

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cophylogeny, Cyprinoidea, Monogenea, phylogeny

1 | INTRODUCTION

Host–parasite coevolution plays an important role in the processes of parasite speciation and represents one of the most fascinating topics in evolutionary biology (Poulin, 2007). If the host specificity of the parasite is high (i.e., a parasite species restricted to a single host species or very few phylogenetically closely related host species), it is tempting to assume that the evolution of parasitic organisms is associated with the evolution of their hosts (Ronquist, 1997). Hence, the Fahrenholz rule (Brooks & McLennan, 1993; Stammer, 1957) states that parasite phylogeny mirrors host phylogeny and that cospeciation drives host–parasite coevolution. Congruent host–parasite phylogenies have usually been inferred when the host switching of parasites is impossible or highly improbable, such as in the case of chewing lice and pocket gophers, where parasite cospeciation likely resulted from an allopatric distribution of hosts and host switching was supported only in the case of physical contact between two gopher species (Hafner & Nadler, 1988; Hafner et al., 1994; Page, 1996). However, the whole concept of the 'Fahrenholz rule' has been re-evaluated and several studies have suggested that cospeciation is not always the predominant driver of parasite speciation during reciprocal host–parasite evolution. Host switching (Klassen, 1992) and parasite duplication, that is parasite speciation within a host lineage (Johnson, Adams, Page, & Clayton, 2003), play significant roles in parasite evolution, often resulting in incongruent host and parasite phylogenies (Desdevises, Morand, Jousson, & Legendre, 2002; Mendlová, Desdevides, Civáňová, Pariselle, & Šimková, 2012; Šimková, Morand, Jobet, Gelnar, & Verneau, 2004; Šimková, Serbielle, Pariselle, Vanhove, & Morand, 2013). Despite the fact that frequent host switching during the evolutionary history of parasite taxa usually results in incongruent host–parasite phylogenies, a series of multiple host switches followed by parasite speciation can generate trees with similar topologies (de Vienne, Giraud, & Shykoff, 2007). Moreover, host switching tends to occur more often between the phylogenetically close host species, what may lead to further congruence between host and parasite trees (Charleston & Robertson, 2002; de Vienne et al., 2013). Therefore, the independent estimation of the age of speciation events in host and parasite trees (e.g., extrapolated from the estimated time of host speciation) should also be taken into account when interpreting the outputs of cophylogenetic analyses.

Dactylogyrus Diesing, 1850 (Monogenea) are gill parasites generally exhibiting narrow host specificity and high morphological variability with respect to attachment organ (termed haptor), putatively reflecting adaptations to their different host species or within-host microhabitats (Gibson, Timofeeva, & Gerasev, 1996; Šimková, Desdevises, Gelnar, & Morand, 2000, 2001; Šimková & Morand, 2008; Šimková, Verneau, Gelnar, & Morand, 2006). In addition, *Dactylogyrus* currently represents the platyhelminth genus with the highest species diversity (more than 900 described species according to Gibson et al., 1996), certainly largely underestimated as new species have recently been described (Aydogdu, Molnár, Emre, & Emre, 2015; Benovics, Kičinjaová, & Šimková, 2017; Nitta & Nagasawa, 2016; Rahmouni, Řehulková, Pariselle, Rkhami, & Šimková, 2017). This high species richness in *Dactylogyrus* is associated with their narrow host specificity towards a single host species or closely related species, and with a high diversity of their host species—primarily freshwater fish of Cyprinoidea (considering recent phylogenetic studies, for example Schönhuth, Vukić, Šanda, Yang, & Mayden, 2018). Previous studies have suggested that each host species harbours at least one *Dactylogyrus* species (Dupont & Lambert, 1986; Galli, Stefani, Zaccara, & Crosa, 2002; Gibson et al., 1996; Moravec, 2001). In regards to host specificity, Šimková, Verneau, et al. (2006) classified five groups of *Dactylogyrus* species ranging from strict specialists, living on a single host species, to generalists parasitizing host species from different phylogenetic lineages. The high host specificity of *Dactylogyrus* (and other monogeneans) is linked with their direct life cycle, where the larva (oncomiracidium) actively searches for a suitable (specific) host and attaches directly to the gills or body surface. Oncomiracidia are sensitive to chemical cues from hosts which can either initiate the hatching of oviparous species, attract larvae or initiate larva deciliation (Buchmann & Lindenstrøm, 2002). The recognition of these signals most likely requires specific parasite adaptation (Buchmann, 1999; Whittington & Kearn, 2011).

Their narrow host specificity and expected host–parasite coevolution make monogeneans potential proxies for the study of the evolution and dispersion of their hosts. Previous studies (on *Lamellodiscus* Johnston & Tiegs, 1922 parasitizing Sparidae, Desdevises et al., 2002; *Gyrodactylus* von Nordmann, 1832 parazitizing Gobiidae, Huyse, Audenaert, & Volckaert, 2003; Huyse, Oeyen, Larmuseau, & Volckaert, 2017; Huyse & Volckaert, 2005; *Cichlidogyrus* Paperna, 1960 and *Scutogyrus* Pariselle & Euzet, 1995 parazitizing Cichlidae, Mendlová et al., 2012; and *Thaparocleidus* Jain, 1952 parasitizing Pangasiidae, Šimková et al., 2013) suggested that cophylogenetic patterns between monogeneans and their hosts are complex, involving less cospeciation than expected and involve putatively high number of host switches, duplications and losses. Frequent host switching in these systems may be expected because of the active dispersion of the larvae and the capacity of adults to survive without the hosts for a short period of time (Bakke, Cable, & Harris, 2007; Brooks & McLennan, 1991), potentially allowing them to infect phylogenetically closely related host species with similar ecological requirements.

In spite of the large interest in host-specific monogeneans, few phylogenetic and/or cophylogenetic studies have been performed for *Dactylogyrus*. In *Dactylogyrus* from central European cyprinoids, intra-host duplication was inferred as a more widespread diversification process than host switching (Šimková et al., 2004).

Several coevolutionary scenarios were proposed by Benovics et al. (2017), Benovics, Desdevises, Vukić, Šanda, and Šimková (2018), and Šimková, Benovics, Rahmouni, and Vukić (2017) regarding *Dactylogyrus* and peri-Mediterranean endemic cyprinoids, the last one hypothesizing that Iberian cyprinids harbour *Dactylogyrus* species originating from two different colonization events.

South European freshwater fauna is extremely rich in endemic cyprinoid species (Kottelat & Freyhof, 2007). For instance, the Balkan Peninsula is considered a hotspot of endemic freshwater diversity and harbours 59% of all European cyprinoid species (Abell et al., 2008; Albrecht & Wilke, 2008; Oikonomou, Leprieur, & Leonardos, 2014; Schultheiss, Albrecht, Bossneck, & Wilke, 2008; Sušnik, Snoj, Wilson, Mrdak, & Weiss, 2007), which have recently become the common interest of ichthyologists (Buj et al., 2017; Gante, 2011; Marková et al., 2010; Perea, Vukić, Šanda, & Doadrio, 2016; Stierandová et al., 2016). The Mediterranean drainages of the Balkans were divided into several ichthyological regions based on the presence of freshwater fish species, especially of the cyprinoids (Oikonomou et al., 2014). The eastern Balkans regions in the Aegean Sea slope are characteristic by the presence of cyprinoid species of Pontocaspian origin (e.g., *Abramis brama*, *Barbus balcanicus*, *Leuciscus aspius* or *Rutilus rutilus* complex), especially in the northern and eastern part (Economidis & Banarescu, 1991; Economou et al., 2007). The conspecifity of these species with populations from the Pontocaspian region was recently genetically corroborated (Geiger et al., 2014; Levin et al., 2017; Marková et al., 2010). Genetic data also suggest affinities of some of the endemic species from this area to Pontocaspian, but also to Anatolian congeners (e.g., *Alburnoides*, *Chondrostoma*, *Squalius*, *Barbus*, *Luciobarbus* or *Vimba*; Geiger et al., 2014; Perea et al., 2010; Stierandová et al., 2016). The south-eastern part of the Balkans, that is south-western part of the Aegean Sea drainages, is on the other hand inhabited by mostly endemic cyprinoids (from genera *Barbus*, *Rutilus*, *Scardinius*, *Telestes* or *Pelasgus*) with affinities to congeneric species from Ionian Sea slope (Buj et al., 2017; Gante, 2011; Geiger et al., 2014; Perea et al., 2010).

The south-western and western part of the Mediterranean drainages of the Balkans is characterized by presence of almost exclusively endemic cyprinoids, both of ancient origin (from Miocene), like genera *Aulopyge*, *Delminichthys*, *Pelasgus*, *Phoxinellus*, or several species of *Telestes* or *Squalius* (Buj et al., 2017, 2019; Gante, 2011; Perea et al., 2010, 2016) as well as of more recent origin, that is species of *Alburnus* or *Scardinius*, probably from Pliocene/Pleistocene colonization events, based on much lower genetic differentiation from congeneric species outside the Balkans (Perea et al., 2010).

In comparison to the species-rich Balkan Peninsula, only several endemic cyprinoid species were described from the Apennine Peninsula (Bianco, 1995). Since most of this peninsula was below the sea level during most of the Miocene era, it is assumed that Apennine ichthyofauna is of more recent origin than ichthyofauna of other south European peninsulas (Steininger & Rögl, 1984). In general, Apennine cyprinoids, especially leuciscids, are phylogenetically more related to Balkan species than to central European or Iberian species (Perea et al., 2010). Several cyprinoid species occur both in the northern part of the Apennine peninsula (northern Adriatic river systems [Padano-Venetian ichthyologic district sensu Bianco, 1990]) and in the western-most Balkan, showing no or very low degree of molecular divergence between taxa from these two regions (Buj et al., 2010; Geiger et al., 2014; Perea et al., 2010). It is a consequence of the glacial periods, when sea level dropped considerably and rivers of the northern Adriatic were connected together, which led to the exchange of many primary native fish species between the two peninsulas (Stefani, Galli, Crosa, Zaccara, & Calamari, 2004; Waelbroeck et al., 2002). However, many of the northern and north-eastern Mediterranean drainages are heavily affected by introductions of non-native freshwater species, including numerous cyprinoids (Bianco, 1995; Piria et al., 2018; Vukić, Eliášová, Marić, & Šanda, 2019), even the endemic ones being translocated often outside the native range (Bianco, 1995; Koutsikos et al., 2019). This could lead to the simultaneous introduction of their non-native parasite species, which can subsequently infect the native fishes (such as parasite *Dactylogyrus*, documented in Benovics et al., 2017).

Since cophylogenetic patterns and processes between peri-Mediterranean cyprinoids and their *Dactylogyrus* parasites are not known, we aimed to study the cophylogeny of these two groups in selected southern European regions in the peri-Mediterranean area and to elucidate the historical dispersion of endemic cyprinoids using *Dactylogyrus* phylogeny. Therefore, the objectives of this study were (a) to reconstruct the coevolutionary histories of Balkan and Apennine endemic cyprinoids and their endemic *Dactylogyrus* parasites, (b) to investigate the speciation patterns of host-specific *Dactylogyrus* and (c) to assess whether parasite phylogeny is linked to host phylogeny and the historical formation of the landmass, or rather to the recent distribution and introduction of non-native species into the investigated regions.

2 | MATERIAL AND METHODS

2.1 | Material collection and fixation

Between 2014 and 2017, 76 cyprinoid species were sampled from 56 localities across the Balkan and Apennine Peninsulas (Table 1). A fin clip was obtained from 608 inspected fish individuals and preserved in 96% ethanol. Fishes were dissected using standard methods described by Ergens and Lom (1970). *Dactylogyrus* parasites were collected from the gills and nasal cavity, mounted on slides, and fixed using a mixture of glycerine and ammonium picrate (GAP, Malmberg, 1957). Species determination was performed according to the size and shape of the sclerotized hard parts of the haptor and the reproductive organs (male copulatory organ and vaginal armament) using Pugachev, Gerasev, Gussev, Ergens, and Khotenowsky (2009). Identification at the species level was performed using an Olympus BX51 microscope equipped with phase-contrast optics. Several representatives of each collected *Dactylogyrus* species were bisected using fine needles. A part of the body (usually the half of body containing the reproductive organs) was mounted on a slide and used

TABLE 1 List of cyprinoid species including localities of their collection

Host species	Country	Locality	Main river basin	Ichthyogeographic district
Alburnoides devolli Bogutskaya, Zupančić & Naseka, 2010	Albania	Devoli, Maliq	Seman	Albanian
Alburnoides economoui Barbieri, Vukić, Šanda & Zogaris, 2017	Greece	Sperchios, Ypati	Sperchios	Western Aegan
Alburnoides fangfangae Bogutskaya, Zupančić & Naseka, 2010	Albania	Osum, Vodice	Seman	Albanian
Alburnoides ohridanus (Karaman, 1928)	Albania	Fani i Vogel, Reps	Mat	Albanian
Alburnoides prespensis (Karaman, 1924)	Greece	Aoos, Kalithea	Aoos	Albanian
Alburnoides strymonicus Chichkoff, 1940	Greece	Angistis, between Alistrati and Drama	Strymon	North-eastern Aegan
Alburnoides thessalicus Stephanidis, 1950	Greece	Pinios, Rongia—Valamandrio	Pinios	North-western Aegan
Alburnus arborella (Bonaparte, 1841)	Italy	Canale maestro de la Chiana, Chuisa dei Capannoi, Arno basis	Arno	Tuscano-Latium
Alburnus neretvae Buj, Šanda & Perea, 2010	Bosnia and Herzegovina	Mušnica, Avtovac	Neretva	Central Adriatic
	Bosnia and Herzegovina	Zagorje, Jabuke	Neretva	Central Adriatic
Alburnus scoranza Bonaparte, 1845	Albania	Skadar lake, Shiroke	Ohrid-Drin-Skadar lake system	Albanian
Aulopyge huegelii Heckel, 1842	Bosnia and Herzegovina	Šujica, Duvansko Polje	Cetina	Central Adriatic
Barbus balcanicus Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002	Greece	Gallikos, Mandres	Gallikos	North-western Aegan
Barbus cyclolepis Heckel, 1837	Greece	Macropotamos River	Filiouri	North-eastern Aegan
Barbus peloponnesius Valenciennes, 1842	Greece	Neda, Gianitsochori	Neda	Ionian
	Greece	Kokitos, Pagrati	Acheron	Ionian
Barbus plebejus Bonaparte, 1839	Croatia	Bribirske Mostine, Bribišnica	Krka	Central Adriatic
Barbus prespensis Karaman, 1924	Albania	Shkumbini, Perrenjas	Shkumbini	Albanian
	Greece	Aoos, Kalithea	Aoos	Albanian
Barbus rebeli Koller, 1926	Albania	Mat, Klos	Mat	Albanian
Barbus sp.	Albania	Kiri	Ohrid-Drin-Skadar lake system	Albanian
Barbus sperchiensis Stephanidis, 1950	Greece	Sperchios, Ypati	Sperchios	Western Aegan
Barbus strumicae Karaman, 1955	Greece	Rihios river, Stavros	Volvi lake	North-eastern Aegan
Barbus tyberinus Bonaparte, 1839	Italy	Torrente Cerfone, Intoppo	Tiber	Tuscano-Latium
Chondrostoma knerii Heckel, 1843	Bosnia and Herzegovina	Rečina river, near Jelim lake, Hutovo Blato	Neretva	Central Adriatic
Chondrostoma ohridana Karaman, 1924	Greece	Aoos, Kalithea	Aoos	Albanian
Chondrostoma phoxinus Heckel, 1843	Bosnia and Herzegovina	Šujica, Šujicko Polje	Cetina	Central Adriatic

(Continues)

TABLE 1 (Continued)

Host species	Country	Locality	Main river basin	Ichthyogeographic district
Chondrostoma vardarense Karaman, 1928	Greece	Angistis, between Alistrati & Drama	Strymon	North-eastern Aegan
	Greece	Pinios, Rongia—Valamandrio	Pinios	North-western Aegan
Delminichthys adspersus (Heckel, 1843)	Bosnia and Herzegovina	Nezdravica, Trebižat	Neretva	Central Adriatic
Luciobarbus albanicus (Steindachner, 1870)	Greece	Trichonis lake, Panetolio	Acheloos	Ionian
Luciobarbus graecus (Steindachner, 1895)	Greece	Sperchios, Ypati	Sperchios	Western Aegan
Pachychilon macedonicum (Steindachner, 1892)	Greece	Pinios, Rongia—Valamandrio	Pinios	North-western Aegan
Pachychilon pictum (Heckel & Kner, 1858)	Albania	Ohrid lake	Ohrid-Drin-Skadar lake system	Albanian
	Greece	Aoos, Kalithea	Aoos	Albanian
Pelasgus laconicus (Kottelat & Barbieri, 2004)	Greece	Evrotas, Sparti	Evrotas	Ionian
Pelasgus marathonicus (Vinciguerra, 1921)†	Greece	Sperchios, Ypati	Sperchios	Western Aegan
Pelasgus stymphalicus (Valenciennes, 1844)†	Greece	Pamissos, Vasiliko	Pamissos	Ionian
Pelasgus thesproticus (Stephanidis, 1939)†	Greece	Acheron, Gliki	Acheron	Ionian
	Greece	Kokitos, Pagrati	Acheron	Ionian
Phoxinellus alepidotus Heckel, 1843	Bosnia and Herzegovina	Bosansko Grahovo, Korana River	Korana	Central Adriatic
Phoxinellus pseudalepidotus Bogutskaya & Zupančić, 2003	Bosnia and Herzegovina	Lištica, Polog	Neretva	Central Adriatic
Phoxinus lumaireul Schinz, 1840†	Croatia	Lovinac, Ričica River	Ričica	Northern Adriatic
Phoxinus sp.	Bosnia and Herzegovina	Zalomka, Ribari	Neretva	Central Adriatic
Protochondrostoma genei (Bonaparte, 1839)	Italy	Torrente Cerfone, Le Ville	Tiber	Tuscano-Latium
Rutilus aula (Bonaparte, 1841)	Croatia	Baštica river, Grabovač reservoir	Baštica	Northern Adriatic
Rutilus basak (Heckel, 1843)	Bosnia and Herzegovina	Krenica lake, Drinovci	Neretva	Central Adriatic
Rutilus lacustris (Pallas, 1814)	Greece	flood pools by Struma, Lithopos	Strymon	North-eastern Aegan
Rutilus ohridanus (Karaman, 1924)	Albania	Skadar lake, Shiroke	Ohrid-Drin-Skadar lake system	Albanian
Rutilus panosi Bogutskaya & Iliadou, 2006†	Greece	Rivio, Amvrakia	Acheloos	Ionian
Rutilus rubilio (Bonaparte, 1837)	Italy	Torrente Cerfone, Intoppo	Tiber	Tuscano-Latium
Rutilus sp.†	Greece	channel near Sperchios	Sperchios	Western Aegan
Scardinius acarnanicus Economidis, 1991†	Greece	Trichonis lake, Panetolio	Acheloos	Ionian
Scardinius dergle Heckel & Kner, 1858	Croatia	Bribirske Mostine, Bribišnica	Krka	Central Adriatic
Scardinius plotizza Heckel & Kner, 1858	Bosnia and Herzegovina	Rečina river, near Jelim lake, Hutovo Blato	Neretva	Central Adriatic
Squalius illyricus Heckel & Kner, 1858	Croatia	Cetina river, Kosore	Cetina	Central Adriatic
Squalius keadicus (Stephanidis, 1971)†	Greece	Evrotas, Sparti	Evrotas	Ionian
Squalius lucumonis (Bianco, 1983)	Italy	Torrente Cerfone, Intoppo	Tiber	Tuscano-Latium

(Continues)

TABLE 1 (Continued)

Host species	Country	Locality	Main river basin	Ichthyogeographic district
Squalius microlepis Heckel, 1843†	Bosnia and Herzegovina	Trebižat, Klobuk	Neretva	Central Adriatic
Squalius orpheus Kottelat & Economidis, 2006	Greece	Rihios river, Stavros	Volvi lake	North-eastern Aegan
Squalius pamvoticus (Stephanidis, 1939)	Greece	Acheron, Gliki	Acheron	Ionian
Squalius peloponensis (Valenciennes, 1844)	Greece	Pamissos, Vasiliko	Pamissos	Ionian
Squalius platyceps upančić, Marič, Naseka & Bogutskaya, 2010	Albania	Ohrid lake	Ohrid-Drin-Skadar lake system	Albanian
Squalius prespensis (Fowler, 1977)	Albania	Shkumbini, Pajove	Shkumbini	Albanian
	Greece	Aoos, Kalithea	Aoos	Albanian
Squalius sp.	Greece	Trichonis lake, Panetolio	Acheloos	Ionian
Squalius squalus (Bonaparte, 1837)	Bosnia and Herzegovina	Donja Drežnica, Drežnica river	Neretva	Central Adriatic
	Italy	Po, Between Verona & Modena	Po	Padano-Venetian
Squalius svallize Heckel & Kner, 1858	Croatia	Konavočica, Grude	Ljuta	central Adriatic
Squalius tenellus Heckel, 1843	Bosnia and Herzegovina	Šujica, Duvansko Polje	Cetina	Central Adriatic
	Bosnia and Herzegovina	Šujica, Šujičko Polje	Cetina	Central Adriatic
Squalius vardarensis Karaman, 1928	Greece	Sperchios, Ypati	Sperchios	Western Aegan
	Greece	Gallikos, Mandres	Gallikos	North-western Aegan
Squalius zrmanjae Karaman, 1928†	Croatia	Udbina, Krbava River	Krbava	Northern Adriatic
Telestes alfiensis (Stephanidis, 1971)	Greece	Erimantos, Tripotamo	Alfios	Ionian
Telestes beoticus (Stephanidis, 1939)†	Greece	stream in Livadia, Kifisos	Kifissos	Western Aegan
Telestes croaticus (Steindachner, 1866)†	Croatia	Sveti Rok, Obsenica river	Obsenica	Northern Adriatic
Telestes dabar Bogutskaya, Zupančić, Bogut & Naseka, 2012	Bosnia and Herzegovina	Vrijeka, Dabarsko Polje	Neretva	Central Adriatic
Telestes fontinalis (Karaman, 1972)	Croatia	Krbavsko polje, Laudonov gaj	Krbava	Northern Adriatic
Telestes karsticus Marčić & Markovčić, 2011	Croatia	Drežnica, Sušik river	Drežnica	Northern Adriatic
Telestes metohiensis (Steindachner, 1901)	Bosnia and Herzegovina	Zalomka River, Nevesinjsko polje	Neretva	Central Adriatic
Telestes montenigrinus (Vukovic, 1963)	Albania	Skadar lake, Shegan	Ohrid-Drin-Skadar lake system	Albanian
Telestes muticellus (Bonaparte, 1837)	Italy	Torrente Cerfone, Intoppo	Tiber	Tuscano-Latium
Telestes pleurobipunctatus (Stephanidis, 1939)	Greece	Kokitos, Pagrati	Acheron	Ionian
Tropidophoxinellus hellenicus (Stephanidis, 1971)†	Greece	Rivio, Amvrakia	Acheloos	Ionian
Tropidophoxinellus spartiaticus (Schmidt-Ries, 1943)	Greece	Neda, Gianitsochori	Neda	Ionian

Note: Host species without *Dactylogyrus* are shown by cross symbol (†).

for morphological identification, while the other part was individually preserved in 96% ethanol for subsequent DNA extraction.

2.2 | DNA extraction, amplification and sequencing

Bisected *Dactylogyrus* samples preserved in ethanol were dried using a vacuum centrifuge. DNA extraction was performed following the standard protocol (DNeasy Blood & Tissue Kit; Qiagen). For molecular analyses, four genetic markers commonly applied for monogeneans were used. A section comprising a part of the 18S rRNA gene, the entire ITS1 region, and partial 5.8S rRNA gene were amplified using the primers S1 (forward, 5′-ATTCCGATAACGAACGAGACT-3′) and IR8 (reverse, 5′-GCTAGCTGCGTTCTTCATCGA-3′), which anneal to the genes for 18S and 5.8S rRNA, respectively (Šimková, Plaisance, Matějusová, Morand, & Verneau, 2003); PCR followed the protocol optimized by Benovics et al. (2018). Partial 28S rRNA gene was amplified using primers C1 (forward, 5′-ACCCGCTGAATTTAAGCA-3′) and D2 (reverse, 5′-TGGTCCGTGTTTCAAGAC-3′) following Hassouna, Michot, and Bachellerie (1984); PCR followed the protocol optimized in Šimková, Matějusová, and Cunningham (2006). The PCR products (~1,000 and ~800 bp, respectively) were checked on 1% agarose gel and purified using the ExoSAP-IT kit (Ecoli) following the standard protocol. The purified products were directly sequenced using the same primers as for PCR and BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

For fish DNA extraction, fin clips were removed from the ethanol and dried, and the JETQUICK Tissue DNA Spin Kit (GENOMED) was applied following the manufacturer's instructions. The complete mtDNA *cytochrome b* gene (1,140 bp) was amplified using primers GluF (forward, 5′-AACCACCGTTGTATTCAACTACAA-3′) and ThrR (reverse, 5′-ACCTCCGATCTTCGGATTACAAGACCG-3′) according to Machordom and Doadrio (2001a). The PCR reaction settings, amplification protocol and PCR product purification followed Šanda et al. (2008). The sequencing was carried out by the Macrogen Service Centre (Seoul, South Korea) using the amplification primers.

The new DNA sequences for parasites and hosts obtained during this study were deposited in GenBank (see Tables S1 and S2 for accession numbers).

2.3 | Phylogenetic reconstruction

DNA sequences of hosts and parasites were aligned using fast Fourier transform in MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002). The new sequences of *Dactylogyrus* were trimmed to concur with the length of sequences obtained from GenBank.

Gaps and ambiguously aligned regions were removed from the alignment of *Dactylogyrus* sequences using GBlocks v. 0.91 (Talavera & Castresana, 2007). The most appropriate DNA evolutionary model was determined using the Bayesian information criterion (BIC) with jModelTest 2.1.10 (Darriba, Taboala, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). Phylogenetic trees were inferred by means of Bayesian inference (BI) and Maximum Likelihood (ML) using MrBayes 3.2 (Ronquist et al., 2012) and RaxML v8.1.X (Stamatakis, 2014), respectively. BI trees were constructed using the Metropolis-coupled Markov chain Monte Carlo algorithm, with two parallel runs of one cold and three hot chains, 10^7 generations, and trees sampled every 100 generations. 30% of all saved trees were discarded as burn-in after checking that the standard deviation split frequency value fell below 0.01. Convergence was assessed using Tracer v.1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). Posterior probabilities (PP) were calculated as the frequency of samples recovering any particular clade. The clade support for ML trees (bootstrap support, BS) was assessed by 1,000 bootstrap pseudoreplicates.

The phylogenetic reconstruction of the relationship between 49 *Dactylogyrus* species was based on combined parts of the genes for 18S and 28S rRNA. The resulting phylogram was rooted by *Dactylogyrus* species from *Carassius gibelio* (Bloch, 1782) and *Cyprinus carpio* L., following Šimková et al. (2004). Data were treated as partitioned and the optimal evolutionary model was selected for each marker individually, including the alpha parameter of the gamma distribution (G) accounting for rate heterogeneity across sites and/or the proportion of invariable sites (I). The phylogenetic reconstruction of the relationship between 76 cyprinoid species based on the complete *cytochrome b* gene was rooted following Mayden et al. (2009), using the outgroup comprising four representatives of the family Cobitidae (*Cobitis jadovaensis* Mustafić & Mrakovčić, 2008 [KP208162], *C. illyrica* Freyhof & Stelbrink, 2007 [KJ487484], *C. narentana* Karaman, 1928 [KP208170] and *C. elongata* Heckel & Kner, 1858 [EF672382]). Host sequence data were treated as codon partitioned, and optimal evolutionary models were selected independently for each position within the codon, including both gamma distribution and the proportion of invariable sites.

2.4 | Cophylogenetic analyses

The tanglegram connecting host and parasite phylogenetic trees via host–parasite associations was built with TreeMap 3.0b (Charleston, 2012). From many existing methods to investigate the congruence between parasite and host phylogenies (de Vienne et al., 2013), a distance-based method and an event-based method were used in the present study. ParaFit (Legendre, Desdevises, & Bazin, 2002), implemented in CopyCat (Meier-Kolthoff, Auch, Huson, & Göker, 2007), was used with patristic distances calculated for each host and parasite phylogeny, and 999 permutations to assess the statistical significance of global and individual coevolutionary links. The event-based analysis was performed with Jane 4.0 (Conow, Fielder, Ovadia, & Libeskind-Hadas, 2010), which allows different costs to be set for each of the five coevolutionary events (i.e., cospeciation, duplication, duplication followed by host switch, loss, and failure to diverge where host speciation is not followed by parasite speciation). Eleven models with different event cost schemes were applied, using 500 generations and a population size of 50 as parameters of the genetic algorithm to assess the influence of each type of evolutionary event. The Jane 4.0 default model, TreeMap default model (Charleston, 1998) and TreeFitter default model (Ronquist, 1995) were included in our analyses following Deng et al. (2013). Each of these default models assumes that cospeciation has the lowest cost (i.e., is the most common evolutionary event). Several additional models were included in the cophylogenetic analyses: TreeFitter models adjusted for host switch and codivergence, respectively; a model with equal weights for coevolutionary events following Mendlová et al. (2012); and five models where each event is alternatively extremely penalized (cost of specific event set to 10 and all others to 1, following Deng et al., 2013). To statistically test whether the global reconstruction cost was significantly lower than expected by chance, 500 randomizations were performed with the use of random parasite trees.

3 | RESULTS

3.1 | Parasite phylogeny

Dactylogyrus parasites were collected from 62 cyprinoid species (Table 1). A total of 49 *Dactylogyrus* species (Table 2) were identified on the basis of morphological markers (Pugachev et al., 2009). Genetic variability was observed among individuals of *Dactylogyrus* species collected from multiple host species and, therefore, all genetic variants were included in the final sequence alignment. The final 1,177 base-pair-long alignment of the 49 putative *Dactylogyrus* species included 138 sequences of partial gene for 18S rRNA combined with partial gene for 28S rRNA (see Supporting Information S3 for alignment). The following optimal evolutionary models were selected: TrNef+I for the 441 bp-long sequence alignment of partial gene for 18S rRNA and TVM+I+G for the 736 bp-long sequence alignment of partial gene for 28S rRNA. BI and ML analyses generated trees with identical topologies (the BI tree is shown in Figure 1). Morphological and molecular data suggested the presence of 10 potentially new species, labelled from *Dactylogyrus* sp. 1 to *Dactylogyrus* sp. 10. The phylogenetic reconstruction divided *Dactylogyrus* species into several groups, of which three were well-supported (A, B and C in Figure 1). The *D. rarissimus* group, which displayed a high level of intraspecific variability (12 genetic variants), formed a sister group to these three large clades, but this group was not supported (PP = 0.49, BS = 51, respectively). The first clade (group A, PP = 0.98, BS = 76) included *D. erhardovae*, *D. cabelleroi* and *D. crucifer*. These three species are common parasites of *Rutilus* spp. The second group (group B, PP = 1, BS = 74) comprised the majority of *Dactylogyrus* species. Within this group, *Dactylogyrus* species were divided into number of lineages of which eight were moderate to well-supported. Different genetic variants of *D. ergensi* collected from six host species from three genera clustered with *D. dirigerus* (a parasite of *Chondrostoma* spp.), *D. caucasicus* and *D. tissensis* (both parasites of *Alburnoides* spp., lineage 1). All four above-mentioned species share a similar shape of male copulatory organ (see Pugachev et al., 2009 for morphology). Each of the four species *D. balkanicus*, *D. dyki*, *D. folkmanovae* and *D. petenyi* contains morphologically similar but genetically different individuals (different genetic forms of the given *Dactylogyrus* species parasitized different host species). However, all different genetic forms of each above-mentioned morphologically identified species did not form monophyletic groups. The well-supported lineage 3 (PP = 1, BS = 100) comprised all genetic variants of *D. dyki*, a common parasite of *Barbus* spp. in Europe, but also included individuals of *D. balkanicus* resulting in the paraphyly of both species. Both *Dactylogyrus* species from *Luciobarbus* (*Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3) formed the well-supported lineage 4. Two potentially new species collected from *C. knerii* and *S. tenellus* (*Dactylogyrus* sp. 4 and *Dactylogyrus* sp. 5, respectively) clustered with *D. nanoides* from *Squalius* spp. and *D. rysavyi*, a known parasite of *Alburnoides* spp. (but collected only from *A. thessalicus* in this study). The phylogenetic proximity of the four above-mentioned species (lineage 5) was well-supported by BI, but only weakly by ML (PP = 0.99, BS = 56). Lineage 6 exclusively comprised potentially new *Dactylogyrus* species collected from *Telestes* spp. (*Dactylogyrus* sp. 6, *Dactylogyrus* sp. 7 and *Dactylogyrus* sp. 8). The monophyly of *D. petenyi* was not supported (lineage 7) because *D. prespensis* clustered with one of the genetic variants of *D. petenyi*. Lineage 8 within group B was formed by *Dactylogyrus* species from *Pachychilon* spp. (PP = 1, BS = 95). The third well-supported group (group C, PP = 1, BS = 91) included *D. alatus*, *D. sphyrna* and *D. vistulae*. All 28 genetic variants of *D. vistulae* collected from 25 cyprinoid species from seven genera formed a well-supported clade (PP = 1, BS = 100).

3.2 | Host phylogeny

The alignment of complete *cytochrome b* sequences was used for phylogenetic analyses of cyprinoid hosts. All investigated cyprinoid species were included in the phylogenetic reconstruction. Five species (*Barbus peloponnesius*, *B. prespensis*, *S. prespensis*, *S. squalus* and *S. vardarensis*) showed interpopulation variability (each cyprinoid species was collected from two localities). One haplotype from each locality for each of these five species was included in the analyses. Additionally, five species (*Alburnus neretvae*, *Chondrostoma vardarense*, *Pachychilon pictum*, *Pelasgus thesproticus* and *S. tenellus*) exhibited no interpopulation variability, even though they were collected from more than one locality, and therefore, only one haplotype from each of these species was included in the analyses, as well as for all other species, which were collected from only one locality. The final alignment contained 85 sequences with 1,140 unambiguous nucleotide positions (see Supporting Information S4 for alignment). GTR+I+G was selected as the best evolutionary model for each position within the codon. Both BI and ML analyses yielded trees with congruent topologies and therefore, only phylogram resulting from BI was used for subsequent analyses (Figure 2). In general, phylogenetic relationships between the respective leuciscid clades (genera) were in congruence with the molecular phylogenies proposed by Perea et al. (2010) and Schönhuth et al. (2018) (e.g., *Telestes* formed well-supported monophyletic group with *Phoxinellus* Heckel, 1843 and

TABLE 2 List of all collected *Dactylogyrus* species and their cyprinoid hosts

Dactylogyrus species	Host species
D. alatus Linstow, 1878	*Alburnus arborella* *Alburnus neretvae*
D. balkanicus Dupont & Lambert, 1986	*Barbus plebejus* *Barbus prespensis* *Barbus rebeli*
D. borealis Nybelin, 1937	*Phoxinus* sp.
D. caballeroi Prost, 1960	*Rutilus ohridanus*
D. caucasicus Mikailov & Shaova, 1973	*Alburnoides devolli* *Alburnoides fangfangae* *Alburnoides prespensis*
D. crivellius Dupont & Lambert, 1986	*Barbus balcanicus* *Barbus peloponnesius* *Barbus plebejus* *Barbus prespensis* *Barbus rebeli* *Barbus* sp. *Barbus tyberinus*
D. crucifer Wagener, 1857	*Rutilus lacustris*
D. difformis Wagener, 1857	*Scardinius plotizza*
D. difformoides Glaeser & Gussev, 1967	*Scardinius plotizza*
D. dirigerus Gussev, 1966	*Chondrostoma ohridana* *Chondrostoma vardarense*
D. dyki Ergens & Lucky, 1959	*Barbus balcanicus* *Barbus cyclolepis* *Barbus peloponnesius* *Barbus prespensis* *Barbus rebeli* *Barbus sperchiensis* *Barbus strumicae*
D. ergensi Molnar, 1964	*Chondrostoma knerii* *Chondrostoma ohridana* *Chondrostoma vardarense* *Protochondrostoma genei* *Squalius lucumonis* *Squalius squalus*
D. erhardovae Ergens, 1970	*Rutilus aula* *Rutilus basak* *Rutilus ohridanus*
D. folkmanovae Ergens,1956	*Squalius* sp. *Squalius orpheus* *Squalius platyceps* *Squalius prespensis* *Squalius squalus* *Squalius vardarensis*
D. ivanovichi Ergens, 1970	*Pachychilon pictum*
D. izjumovae Gussev, 1966	*Scardinius dergle* *Scardinius plotizza*
D. martinovici Ergens, 1970	*Pachychilon pictum*
D. minor Wagener, 1857	*Alburnus scoranza*
D. nanoides Gussev, 1966	*Squalius prespensis* *Squalius squalus*
D. nanus Dogiel & Bychowsky, 1934	*Rutilus rubilio*
D. omenti Benovics et al., 2017	*Aulopyge huegelii*
D. parvus Wegener, 1910	*Alburnus scoranza*
D. petenyi Kastak, 1957	*Barbus balcanicus* *Barbus cyclolepis* *Barbus peloponnesius*
D. petkovici Ergens, 1970	*Pachychilon pictum*
D. prespensis Karaman, 1924	*Barbus prespensis*
D. prostae Molnar, 1964	*Squalius* sp. *Squalius lucumonis* *Squalius pamvoticus* *Squalius prespensis* *Squalius squalus*
D. rarissimus Gussev, 1966	*Alburnus arborella* *Alburnus neretvae* *Pelasgus laconicus* *Rutilus basak* *Rutilus lacustris* *Rutilus ohridanus* *Rutilus rubilio* *Telestes alfiensis* *Telestes dabar* *Telestes fontinalis* *Telestes metohiensis*
D. rosickyi Ergens, 1970	*Pachychilon pictum*
D. rutili Glaeser, 1965	*Rutilus basak* *Rutilus lacustris* *Rutilus ohridanus*
D. rysavyi Ergens, 1970	*Alburnoides thessalicus*
D. sekulovici Ergens, 1970	*Pachychilon pictum*
D. soufii Lambert, 1977	*Telestes montenigrinus*
Dactylogyrus sp. 1	*Delminichthys adspersus*
Dactylogyrus sp. 2	*Luciobarbus graecus*
Dactylogyrus sp. 3	*Luciobarbus albanicus*
Dactylogyrus sp. 4	*Chondrostoma knerii*
Dactylogyrus sp. 5	*Squalius tenellus*
Dactylogyrus sp. 6	*Telestes karsticus*
Dactylogyrus sp. 7	*Telestes muticellus*

(Continues)

TABLE 2 (Continued)

Dactylogyrus species	Host species
Dactylogyrus sp. 8	*Telestes montenigrinus*
Dactylogyrus sp. 9	*Tropidophoxinellus spartiaticus*
Dactylogyrus sp. 10	*Pachychilon macedonicum*
D. sphyrna Linstow, 1878	*Rutilus basak*
	Rutilus ohridanus
	Rutilus rubilio
D. suecicus Nybelin, 1937	*Rutilus lacustris*
D. tissensis Zachvatkin, 1951	*Alburnoides thessalicus*
D. vastator Nybelin, 1924	*Aulopyge huegelii*
	Barbus plebejus
D. vistulae Prost, 1957	*Alburnoides ohridanus*
	Alburnoides strymonicus
	Alburnoides thessalicus
	Chondrostoma ohridana
	Chondrostoma phoxinus
	Chondrostoma vardarense
	Phoxinellus alepidotus
	Phoxinellus pseudalepidotus
	Protochondrostoma genei
	Rutilus rubilio
	Squalius illyricus
	Squalius lucumonis
	Squalius peloponensis
	Squalius platyceps
	Squalius prespensis
	Squalius squalus
	Squalius svallize
	Squalius tenellus
	Squalius vardarensis
	Telestes fontinalis
	Telestes karsticus
	Telestes metohiensis
	Telestes montenigrinus
	Telestes muticellus
	Telestes pleurobipunctatus
D. vranoviensis Ergens, 1956	*Squalius squalus*
	Squalius vardarensis
D. yinwenyingae Gussev, 1962	*Squalius lucumonis*

Chondrostoma s.l. Agassiz, 1832; *Delminichthys* Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig, 2006 and *Pelasgus* Kottelat & Freyhof, 2007 formed well-supported group, and *Phoxinus* Rafinesque, 1820 clade displayed a sister position to other leuciscids). Tribus Barbini (Cyprinidae) formed a strongly supported group in the sister position to the leuciscids. However, the clade of the genus *Barbus* was only weakly supported by both analyses (PP = 0.68, BS = 56). Using the present data set, *A. huegelii* appears in sister position to *Luciobarbus* spp.

3.3 | Cophylogeny

BI phylogenetic reconstructions were used for cophylogenetic analyses (Figure 3). The distance-based analysis using ParaFit yielded a highly significant ($p < .001$) overall cophylogenetic structure. Out of 138 host–parasite individual links, 65 contributed significantly to the global cophylogenetic structure ($p < .05$). Significant links ($p < .05$) were inferred between the representatives of group C (*D. alatus*, *D. sphyrna* and *D. vistulae* or their genetic variants, Figure 1) and their leusiscid host species, and between *Dactylogyrus* representatives belonging to lineage 8 (*D. martinovici*, *D. petkovici* and *Dactylogyrus* sp. 10) and their *Pachychilon* hosts. Highly significant individual links ($p < .001$) were found between representatives of the cyprinid genera *Barbus* and *Luciobarbus* and the monotypic *Aulopyge* and their *Dactylogyrus* spp. (or genetic forms of these *Dactylogyrus*): '*D. balkanicus*', *D. crivellius*, '*D. dyki*', '*D. petenyi*', '*D. prespensis*' from *Barbus*, undescribed *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3 from *Luciobarbus* spp., and *D. omenti* from *A. huegelii*. Subsequent analysis performed using the same number of permutations (999) and focussed only on this group supported the initial significant cophylogenetic structure ($p < .05$).

Applying different cost schemes, Jane produced reconstructions with similar proportions of coevolutionary events (Table 3). Global costs using each scheme were all statistically significant ($p < .01$). In general, it appears that *Dactylogyrus* speciation is primarily driven by duplication followed by host switching, which was an important component in 8 of the 11 models tested. The lowest total cost was produced by the host switch-adjusted TreeFitter model. The duplication-prohibited model and host switch-prohibited model resulted in a high number of loss events and represented the scenarios with the highest total costs (also suggesting the importance of host switching in the evolution of *Dactylogyrus*). Setting the duplication cost to zero and equalizing the costs of the other events (codivergence adjusted TreeFitter model) or extremely penalizing cospeciation cost (cospeciation-prohibited model) resulted in a higher occurrence of duplication events compared to cospeciation events in contrast to a relatively low occurrence of duplication events within each of the other models. Additionally, no losses were inferred in these models (models 4, 6 and also 9, Table 3). A high number of cospeciations were inferred in models with the cospeciation cost set to zero or in models with a high penalization of duplication, host switching or failure to diverge. A low occurrence of duplication events was found either when cospeciation was not penalized (TreeMap default model), or when failure to diverge or duplication were highly penalized (FTD prohibitive model and duplication prohibitive models, respectively). In the latter model, a remarkably high number of losses were inferred (such as in the case of the host switch-prohibited model).

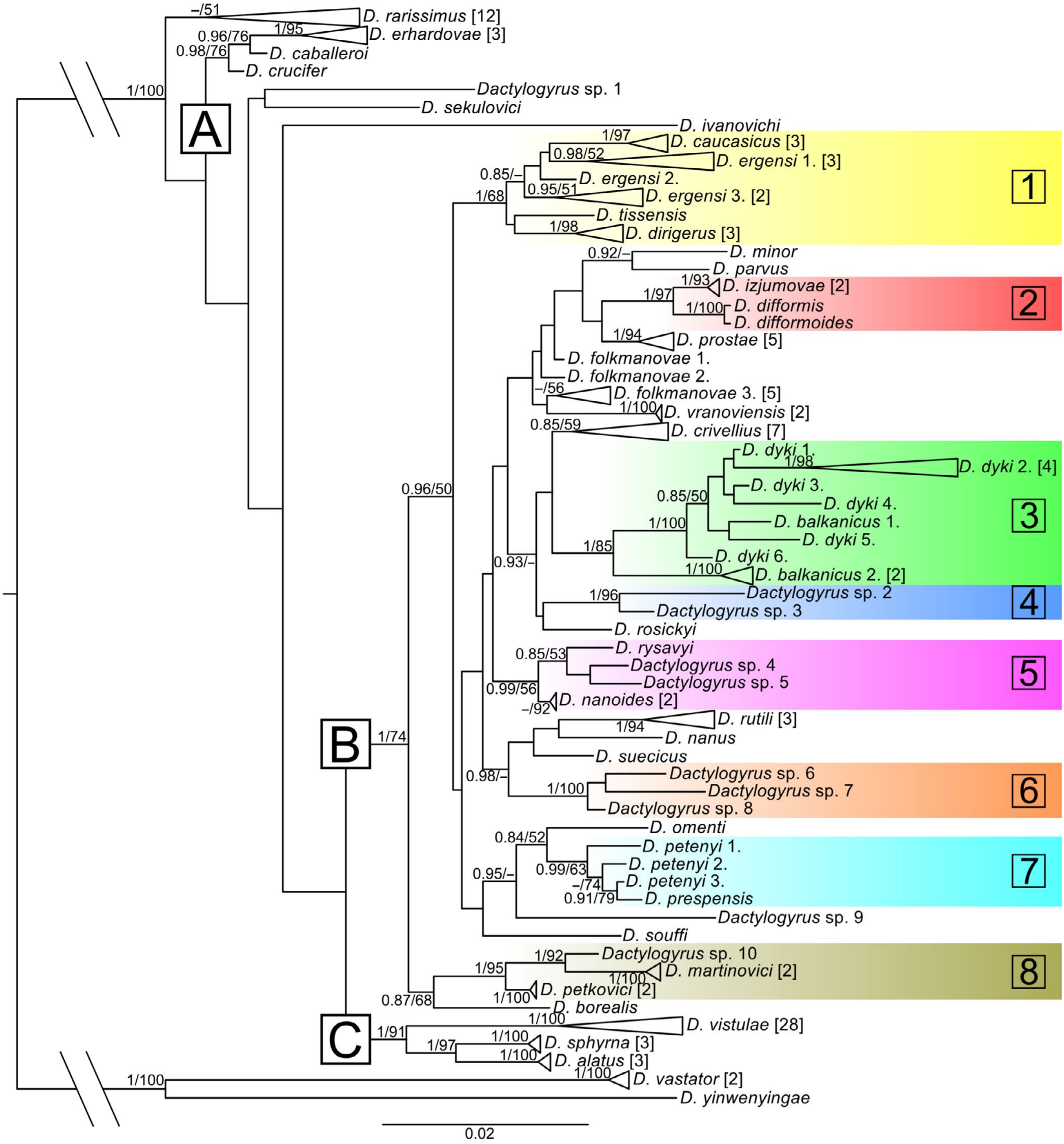

FIGURE 1 Phylogenetic tree of 139 haplotypes from 49 *Dactylogyrus* species collected in the Balkan and Apennine Peninsulas reconstructed by Bayesian inference (BI). The tree is based on combined partial sequences of genes coding 18S rRNA and 28S rRNA. Values among branches indicate posterior probabilities from BI and bootstrap values from ML analyses. Values below 0.80 (BI) and 50 (ML) are shown as dashes. Branch lengths represent the number of substitutions per site. Letters in boxes (A–C) and numbers in the coloured areas (1–8) represent specific and well-supported lineages described in Section 3. Numbers of genetic variants within each collapsed group are shown in brackets

Applying the same cost schemes with the same number of generations and population size on a selected subgroup of cyprinids belonging to the Barbini tribe and their respective specific *Dactylogyrus* spp., between which a strong cophylogenetic signal was initially detected, resulted in only five schemes yielding cophylogenetic scenarios with statistically significant global costs (tested on 500 randomizations, Table 4). Three of these five models (schemes 1, 3 and 6) were set to expect duplication followed by host switching as the least probable coevolutionary event simulating the allopatric speciation of hosts where the host switching of

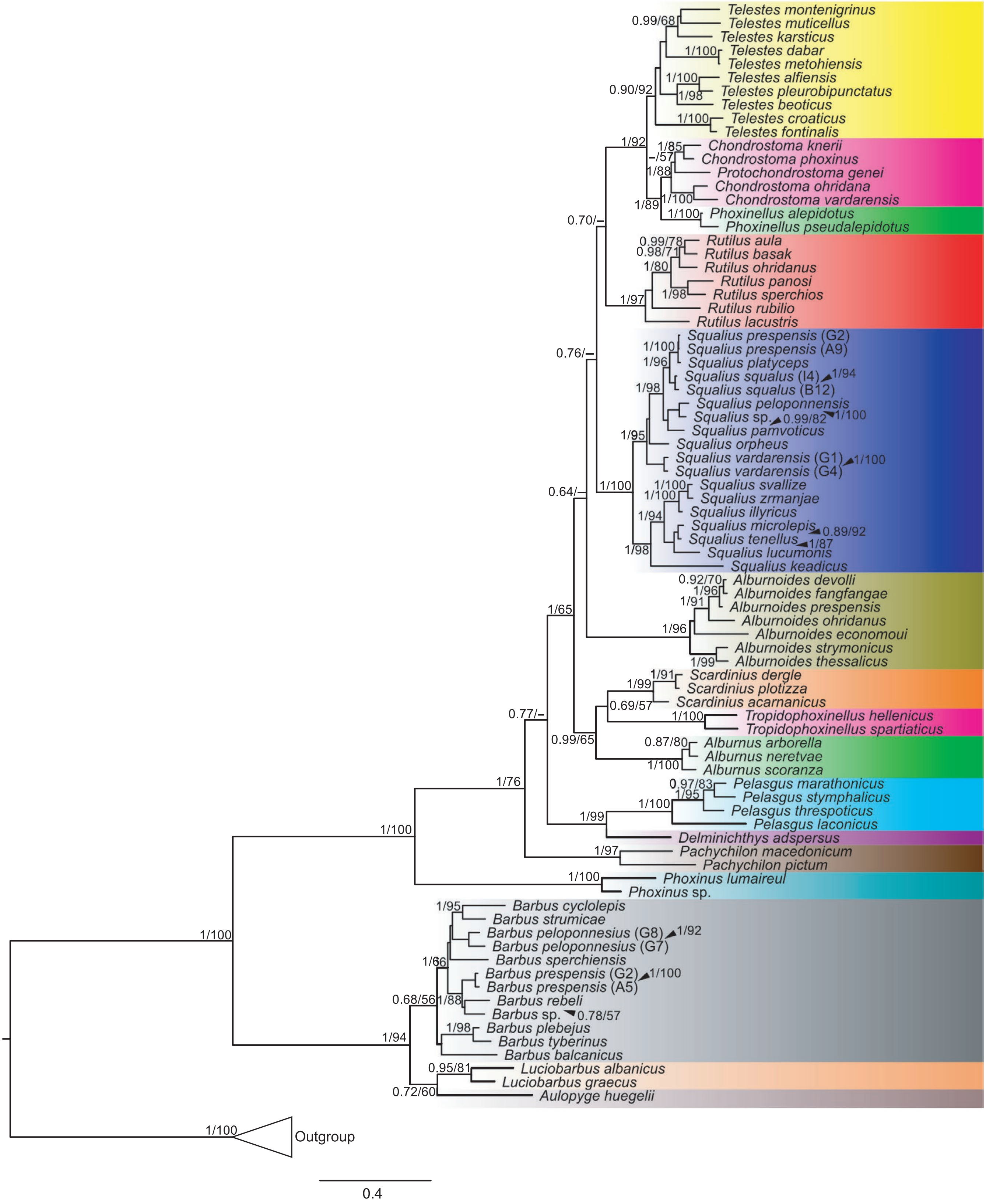

FIGURE 2 Phylogenetic tree of 85 haplotypes belonging to 76 endemic cyprinoid species from the Balkan and Apennine Peninsulas, reconstructed by Bayesian inference (BI). The tree is based on 1,140 bp-long complete *cytochrome b* sequences and rooted using four representatives of the family Cobitidae. Values among branches indicate posterior probabilities from BI and bootstrap values from ML analyses. Values below 0.60 (BI) and 50 (ML) are shown as dashes. Branch lengths represent the number of substitutions per site. Coloured areas represent clades comprising individual genera

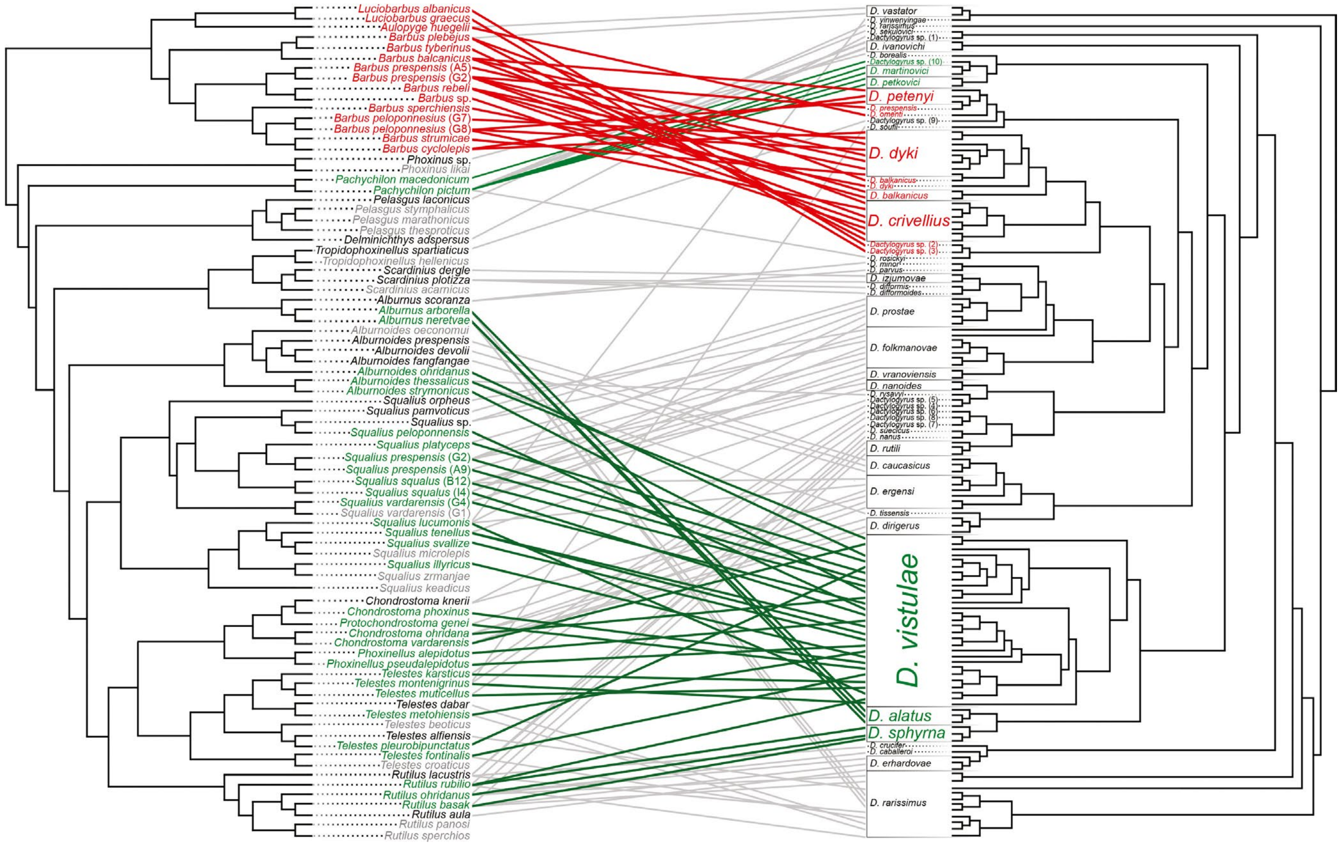

FIGURE 3 Tanglegram showing the associations between Cyprinoidea (left) and their *Dactylogyrus* parasites (right). Phylogenetic trees were reconstructed by Bayesian inference (Figures 1 and 2). Coloured lines represent statistically significant links computed with ParaFit (green $p < .05$; red $p < .001$). Each bracket represents the haplotypes belonging to one *Dactylogyrus* species. Host taxa without *Dactylogyrus* are shown in grey

parasites between new lineages is unlikely (an example of a cophylogenetic scenario from this subsequent data set is presented in Figure 4). Nevertheless, in the majority of scenarios, duplication followed by host switching was the most common coevolutionary event. This event was omitted only in the case of its extremely high penalization, modelling the scenario where physical contact between congeneric host species should be completely excluded. Equalizing all event costs, or highly penalizing other coevolutionary events when compared to duplication followed by host switching resulted in the same proportions of coevolutionary events. However, the results of all models with these cost schemes were not statistically significant.

4 | DISCUSSION

4.1 | Phylogeny of *Dactylogyrus*

Following the former phylogenetic study by Benovics et al. (2018) focussed on 53 *Dactylogyrus* species parasitizing endemic cyprinoids in the Balkans, this work is the first wide-ranging study focussing on the cophylogenetic relationships between endemic cyprinoids of the north-eastern European peri-Mediterranean and their specific parasites. In the present study, a large data set of 76 endemic cyprinoid species covering 95% of the known cyprinoid diversity of the whole north-eastern European peri-Mediterranean region (Balkan and Apennine Peninsulas) was used. A total of 49 morphologically identified *Dactylogyrus* species were recognized, representing 139 genetic variants. In the majority of host–parasite associations, *Dactylogyrus* species were specific to a single cyprinoid species or to a group of congeneric cyprinoids. For many *Dactylogyrus* species parasitizing several cyprinoid species, that is generalists, different genetic variants of morphologically identical *Dactylogyrus* species were observed. In the majority of cases, even these genetic variants exhibited host specificity—unique genetic variant was found in a single host species.

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

Group B, recognized from phylogenetic reconstruction, contained several well-to-moderately supported clades. However,

TABLE 3 Outputs of cophylogenetic analyses calculated using 11 models with different cost schemes

Model	Event costs	Total cost	Cospeciation	Duplication	Duplication and Host switch	Loss	Failure to diverge
Jane default†	0 1 2 1 1	220	58	15	64	77	–
TreeMap default†	0 1 1 1 1	120	46	7	84	29	–
TreeFitter default†	0 0 2 1 1	183	39	18	80	23	–
Codivergence adjusted TreeFitter model†	1 0 1 1 1	116	–	21	116	–	–
Host switch-adjusted TreeFitter model†	0 0 1 1 1	100	29	14	94	6	–
Cospeciation prohibitive†	10 1 1 1 1	137	–	21	116	–	–
Duplication prohibitive†	1 10 1 1 1	399	72	10	55	172	–
Host switch prohibited†	1 1 10 1 1	588	70	56	11	352	–
Sorting prohibited†	1 1 1 10 1	137	18	17	102	–	–
FTD prohibitive†	1 1 1 1 10	142	28	14	95	5	–
Equal weights†	1 1 1 1 1	144	30	14	93	7	–

Note: Total costs represent the sum of inferred numbers of each evolutionary event multiplied by their respective costs. Values in columns represent frequency of the specific evolutionary event in the reconstruction resulting from applied scheme. Statistically significant scenarios are marked by cross symbol (†). Dashes (–) represent null values.

TABLE 4 Outputs of cophylogenetic analyses calculated using 11 models with different cost schemes applied to subset of cyprinids from the tribe Barbini and their respective *Dactylogyrus* species

Model	Event costs	Total cost	Cospeciation	Duplication	Duplication and Host switch	Loss	Failure to diverge
Jane default†	0 1 2 1 1	37	13	1	16	4	–
TreeMap default†	0 1 1 1 1	21	12	1	17	3	–
TreeFitter default†	0 0 2 1 1	36	13	2	15	6	–
Codivergence adjusted TreeFitter model	1 0 1 1 1	26	–	4	26	–	–
Host switch-adjusted TreeFitter model†	0 0 1 1 1	20	12	1	17	3	–
Cospeciation prohibitive	10 1 1 1 1	30	–	2	28	–	–
Duplication prohibitive	1 10 1 1 1	30	–	2	28	–	–
Host switch prohibited†	1 1 10 1 1	90	17	13	–	60	–
Sorting prohibited	1 1 1 10 1	30	–	2	28	–	–
FTD prohibitive	1 1 1 1 10	30	–	2	28	–	–
Equal weights	1 1 1 1 1	30	–	2	28	–	–

Note: Total costs represent the sum of inferred numbers of each evolutionary event multiplied by their respective costs. Values in columns represent frequency of the specific evolutionary event in the reconstruction resulting from applied scheme. Statistically significant scenarios are marked by cross symbol (†). Dashes (–) represent null values.

several *Dactylogyrus* species, formerly recognized on the basis of morphology, were not phylogenetically supported as monophyletic. These species include *D. ergensi*, *D. folkmanovae*, *D. dyki*, *D. balkanicus* and *D. petenyi*. The monophyly of *D. ergensi* was not supported, as *D. caucasicus* collected from *Alburnoides* spp. was included in a well-supported group comprising all *D. ergensi* individuals. However, two well-supported groups that follow the biogeographical distribution of leuciscid hosts were formed by *D. ergensi* individuals (Figure 1). *Dactylogyrus ergensi* lineage 1, a sister group to *D. caucasicus*, included individuals found on *Protochondrostoma genei*, *S. lucumonis* and *S. squalus*, all leuciscid species native to the central/northern Adriatic and neighbouring Tyrrhenian ichthyogeographic districts (Bianco, 1990). The other clade, *D. ergensi* lineage 3, contained the genetic forms of *D. ergensi* collected from *C. ohridana* and *C. vardarense*, both endemic to the southern Balkans, specifically to the Albanian and north-eastern Aegan ichthyogeographic districts (Kottelat & Freyhof, 2007). The present data suggest that *D. ergensi* encompasses several species. In fact, the morphometric variability in the shape and size of the male copulatory organ of *D. ergensi* from the *Chondrostoma* spp. in different regions of Europe was reported in its original description by Gussev (1966). Later, Lambert (1977) proposed the splitting of *D. ergensi* by separating *D. toxostomi* (parasitizing *Parachondrostoma toxostoma* (Vallot, 1837)), but its taxonomic status was not

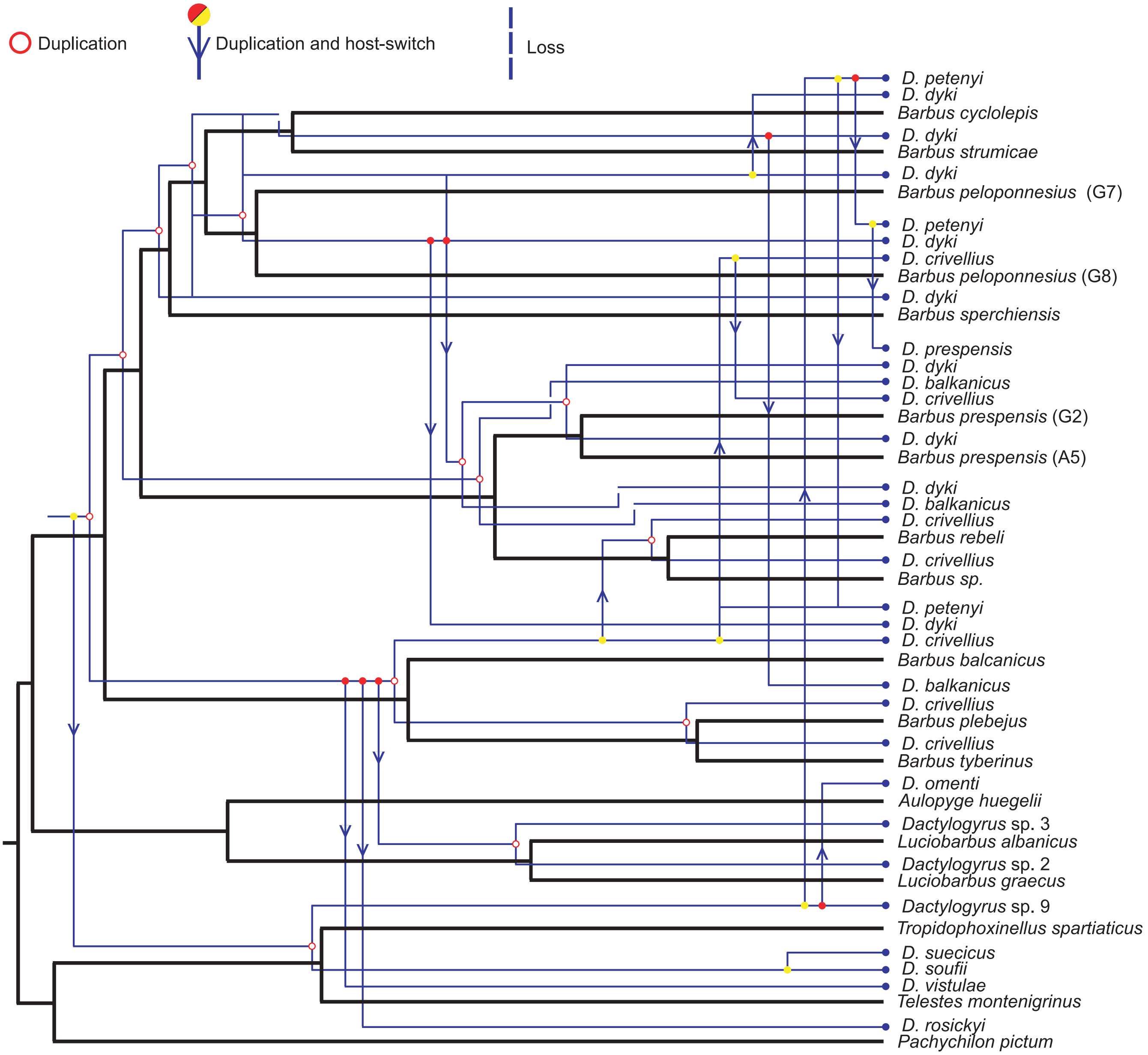

FIGURE 4 One of the possible cophylogenetic scenario between representatives of the tribe Barbini and their specific *Dactylogyrus* species constructed with Jane 4.0 (11 cospeciations, 1 duplication, 18 duplications followed by host switch, 4 losses and 0 failure to diverge). Black branches represent the host phylogeny and blue branches represent the parasite phylogeny. Red and yellow circles represent host switching of the parasite

considered valid since measurements of the sclerotized parts of the attachment organ and male copulatory organ overlapped with *D. ergensi* individuals (Pugachev et al., 2009). Therefore, on the basis of the present molecular data, we can conclude that *D. ergensi*, originally described as a parasite of *Chondrostoma* spp. (although its presence was also documented on *Squalius* spp. in the Apennines), is in fact a species complex. Our results also suggest that *D. caucasicus* evolved from *D. ergensi* by host switching to the phylogenetically distant *Alburnoides* Jeitteles, 1861 species (Perea et al., 2010; Schönhuth et al., 2018), moreover both of these *Dactylogyrus* species have a similar shape with respect to the male copulatory organs (Pugachev et al., 2009).

The previous phylogenetic reconstruction of *Dactylogyrus* performed by Šimková et al. (2004) was focussed on the species parasitizing central European cyprinoids. Our study confirmed most of the phylogenetic relationships between *Dactylogyrus* species previously suggested in their study. For example, the sister species *D. minor* and *D. parvus* parasitizing *A. alburnus* L. in Central Europe were also found on *A. scoranza* in the Balkans. *Dactylogyrus izjumovae*, *D. difformis* and *D. difformoides*, all parasites of *Scardinius erythrophthalmus* L. in Central Europe, formed a monophyletic group also reported in the phylogenetic reconstruction of *Dactylogyrus* parasitizing endemic Balkan leuciscids, more specifically *S. plotizza* and *S. dergle*. Congruency was also reported in the sister position of *D. prostae* of the clade formed by *Dactylogyrus* from *Scardinius* Bonaparte, 1837. The present results suggest that *D. nanoides* is phylogenetically closer to the new *Dactylogyrus* species from *Chondrostoma knerii* and *S. tenellus* (*Dactylogyrus* sp. 4 and sp. 5 respectively) and to *D. rysavi* rather than to *D. folkmanovae* (as was shown in the phylogenetic reconstruction of *Dactylogyrus* parasitizing Central European cyprinoids by Šimková et al., 2004). However, *D. folkmanovae* collected from seven *Squalius* species appears to be paraphyletic, as its representatives clustered with other *Dactylogyrus* from leuciscids including *D. prostae* and *D. vranoviensis* parasitizing *Squalius*, which also suggests the existence of a *D. folkmanovae* morphotype species complex. The phylogenetic position of *D. borealis* is very interesting, as this species is host-specific only for representatives of the genus *Phoxinus* in the Balkans and Central Europe. According to Šimková et al. (2004), *D. borealis* is phylogenetically proximal to *D. amphibothrium* Wagener, 1857 and *D. hemiamphibothrium* Ergens, 1956, both parasitizing *Gymnocephalus cernuus* L. (Percidae) in the Czech Republic. However, considering only *Dactylogyrus* of cyprinoids (more specifically only leuciscids in our study), *D. borealis* clusters together with *Dactylogyrus* spp. of *Pachychilon* Steindachner, 1882 (*Dactylogyrus* lineage 8), which is endemic in the Balkans and represents the ancient leuciscid lineage in this region.

The high molecular diversity among *Dactylogyrus* individuals collected from three *Telestes* species (*T. karsticus*, *T. muticellus* and *T. montenigrinus*) suggests the existence of three unknown *Dactylogyrus* species (*Dactylogyrus* sp. 6, sp. 7 and sp. 8 respectively, representing *Dactylogyrus* lineage 6). Extrapolating from the branch lengths and molecular similarity, we can postulate that these species diverged probably by cospeciation with the *Telestes* genus (see phylogeny in Buj et al., 2017). On the basis of the shape and size of sclerotized elements of the haptor and copulatory organs, these three potentially new species greatly resemble *D. nanus* and *D. suecicus*, belonging together with *D. rutili* to the clade which is sister to the clade including three new *Dactylogyrus* species parasitizing *Telestes*. *Dactylogyrus nanus*, *D. rutili* and *D. suecicus* are common parasites of *Rutilus*, the leuciscid species which is phylogenetically related to *Telestes* (Perea et al., 2010; Schönhuth et al., 2018, and also supported by our results, see below).

The group C, also recognized in previous phylogenetic studies (Benovics et al., 2018; Šimková et al., 2004), was strongly supported in the present study. It comprises *D. alatus*, *D. sphyrna* and *D. vistulae*, which all possess large haptoral anchor hooks (‘sphyrna’ morphotype) and miss a ventral connective bar except for *D. alatus*, which has a thin ‘phoxini’ type ventral connective bar (Pugachev et al., 2009). Šimková et al. (2004) also suggested that *Dactylogyrus similis* Wagener, 1909, morphologically close to *D. sphyrna* and *D. vistulae*, is included in this group, but this species was not found on endemic cyprinoids of the north-eastern peri-Mediterranean region. While *D. alatus* and *D. sphyrna* were collected from two *Alburnus* Rafinesque, 1820 and three *Rutilus* Rafinesque, 1820 species, *D. vistulae* used a wide range of host species representing different genera and exhibiting a wide biogeographical distribution. However, the true origin of this generalist species is unknown and to investigate it we suggest that the representatives from Central European cyprinoids (e.g., *Squalius cephalus* L. or *Chondrostoma nasus* L.), in which molecular variability was also observed (Šimková et al., 2004), should be included in future studies, based on population genetic markers (necessary to be developed).

4.2 | Phylogeny of Cyprinoidea

The phylogenetic reconstruction of the north-eastern peri-Mediterranean leuciscids obtained in this study is in general agreement with the molecular phylogenies proposed by Perea et al. (2010) and Schönhuth et al. (2018). Observed differences in the resulting generic phylogenies are most probably due to different taxon sampling, limited in the case of our study to the Balkan and Apennine representatives, and in comparison with Schönhuth et al. (2018) also in different markers used (multilocus study). Basically, all genera were resolved in our study as monophyletic, with exception of *Chondrostoma*, which in our study include *Protochondrostoma*. This is the most probably a result of limited taxon sampling in our study. Genus *Protochondrostoma* was defined by Robalo, Almada, Levy, and Doadrio (2007), together with *Achondrostoma*, *Iberochondrostoma*, *Parachondrostoma* and *Pseudochondrostoma* (all from Iberian peninsula), which are not included in our phylogenetic reconstruction.

Our study supports the phylogenetic grouping of *Alburnus*, *Scardinius* and *Tropidophoxinellus*, which was previously hypothesized (Briolay, Galtier, Brito, & Bouvet, 1998; Brito, Briolay, Galtier, Bouvet, & Coelho, 1997; Perea et al., 2010; Zardoya & Doadrio, 1999). Interestingly, all three genera harbour *Dactylogyrus* from different evolutionary lineages. While *Alburnus* spp. are parasitized by *D. alatus*, *D. minor*, *D. parvus* and *D. rarissimus* (the last is a common species on *Rutilus* spp. and *Telestes* spp. and rare on *Pelasgus* spp.), *Scardinius* and *Tropidophoxinellus* harbour host-specific *Dactylogyrus* spp. (*D. difformis*, *D. difformoides*, *D. izjumovae* and *Dactylogyrus* sp. 9). The phylogenetic relationships within the *Alburnoides* clade follow the biogeographical distribution of *Alburnoides* species: a clade formed by *A. ohridanus*, *A. prespensis*, *A. devolli* and *A. fangfangae* comprises species distributed in the Albanian ichthyogeograpical district (Kottelat & Freyhof, 2007), and a second clade is formed by *A. strymonicus* and *A. thessalicus* from the Aegan district.

Regarding the cyprinids, in our phylogenetic reconstruction, the genus *Barbus* was supported only weakly, however, it formed a monophyletic clade. In the present study, *A. huegelii* seems to be phylogenetically closer to the *Luciobarbus* clade, although this relationship is only moderately supported. The phylogenetic position of *A. huegelii* appears generally uncertain. Yang et al. (2015) suggested that *A. huegelii* occupied the sister position to *Barbus* lineage, while Gante (2011) showed its sister position to clade comprising both *Barbus* and *Luciobarbus* genera.

4.3 | Cophylogenetic host–parasite relationships

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

It has been hypothesized that during evolutionary time monogeneans developed very specialized haptors specifically to attach to (generally one) well-defined host species (Jarkovský, Morand, Šimková, & Gelnar, 2004; Sasal, Trouvé, Müller-Graf, & Morand, 1999; Šimková, Desdevises, Gelnar, & Morand, 2001). For example, Šimková et al. (2001) found a positive correlation between the size of *Dactylogyrus* anchor hooks and the size of their host species. Such highly adapted attachment organs would make the switch to a different host species very difficult, and even unlikely (but that may depend on the intraspecific variability of the sclerified pieces in this organ, see Kaci-Chaouch, Verneau, & Desdevises, 2008). However, some *Dactylogyrus* species, such as *D. vistulae*, parasitize phylogenetically distant hosts, from small-sized (e.g., *Alburnoides* spp. or *Phoxinellus* spp.) to large-sized species (e.g., *Chondrostoma* spp. or *Squalius* spp.), displaying only minor morphological variability in their haptoral sclerites (M. Benovics, unpublished data). This species clusters among the largest *Dactylogyrus* species (see Pugachev et al., 2009 for morphology), exhibiting also large anchor hooks, which suggests that monogenean species developing large attachment structures as an adaptation to large-sized hosts can host switch to smaller-size hosts.

According to our results, host switching clearly appears to be the main coevolutionary event inferred from the cophylogenetic reconstructions of *Dactylogyrus* and their hosts, followed by cospeciation (Table 3). Host switches likely result here from the sympatric distribution of phylogenetically distant cyprinoid species linked to the historical shift of the landmass and/or from the more recent human-induced introduction of non-native cyprinoid species into the Balkans and Apennines. In the present study, intra-host speciation (i.e., duplication) is suggested to be a rather rare coevolutionary event. This is in contrast to previous cophylogenetic studies on dactylogyrids, where intra-host duplication was the most commonly inferred coevolutionary event (e.g., *Dactylogyrus* by Šimková et al., 2004, *Cichlidogyrus* and *Scutogyrus* on cichlids by Mendlová et al., 2012, or *Thaparocleidus* on pangasiids by Šimková et al., 2013). This may be explained by the fact that these studies included either a limited number of host species from the investigated area or a high number of representatives from phylogenetically distant host species where host switching was highly improbable, in contrast to our study where highly diversified groups of phylogenetically close and/or sympatric cyprinoid species were included. This suggests that host switching is the primary cause of speciation in *Dactylogyrus*, followed by intra-host speciation only if host switching is not possible due to geographical isolation or phylogenetic divergence (then presenting too large differences in parasites' microhabitat) among fish species living in sympatry.

In the present study, a statistically significant overall cophylogenetic structure was inferred among *Dactylogyrus* and their Cyprinoidea hosts. The significant global fit computed with ParaFit relies on 47% significant individual host–parasite links. Among these individual associations, the most significant were found between cyprinids of the Barbini tribe and their *Dactylogyrus* spp. All these *Dactylogyrus* species are genus-specific and their phylogenetic relationships followed the evolutionary history of barbels. However, this *Dactylogyrus* group is potentially subjected to cospeciation, as suggested in testing different cost schemes and reconstructing scenarios from phylogenetic trees topologies. Cophylogenetic analyses considering only fish in Barbini and their *Dactylogyrus* species confirmed this significant cophylogenetic structure and suggested scenarios strongly implying duplication events in the evolutionary history of *Dactylogyrus* from Barbini. This intimate coevolutionary history between 'barbels' and their specific *Dactylogyrus* lineages could be related to the fact that Barbini belong to another group, Cyprinidae (Machordom & Doadrio, 2001b; Schönhuth et al., 2018; Yang et al., 2015). We can hypothesize that during evolution several *Dactylogyrus* species (i.e., *D. balkanicus*, *D. dyki*, *D. crivellius*) specialized on barbels, as is supported also by their specific distribution on European *Barbus* and the strong cophylogenetic structure between *Dactylogyrus* and Barbini in the Balkan and Apennine Peninsulas (Figure 4). However, two species, *D. petenyi* and *D. prespensis* (representatives of *Dactylogyrus* lineage 7 in our phylogenetic reconstruction), likely colonized their host via a recent host switching from phylogenetically distant cyprinoid taxa, followed by fast speciation on endemic barbels.

In addition to *D. vistulae*, a strong cophylogenetic signal was also inferred between *D. alatus* and *D. sphyrna*, each with their respective hosts. In central Europe, these two species parasitize hosts from two or more leuciscid genera (Moravec, 2001), while in southern European peninsulas, they use only *Alburnus* spp. and *Rutilus* spp., respectively. Frequent host switching in the evolutionary history of these *Dactylogyrus* species, inferred by the event-based analyses in Jane, suggest that these species originally parasitized *Alburnus* and *Rutilus*, and subsequently switched to other leuciscid genera in central Europe.

The cophylogenetic history of *Pachychilon* and their *Dactylogyrus* parasites reconstructed in this study is noteworthy. Despite the fact that all *Dactylogyrus* species are genus or species-specific, they in this case do not form a monophyletic group. Three of the six *Dactylogyrus* species from *Pachychilon* spp. found in this study formed a clade within group B (lineage 8), and a strong cophylogenetic signal was observed exclusively between these species and their representative *Pachychilon* hosts. This suggests that *D. petkovici* and the common ancestor of *D. martinovici* and *Dactylogyrus* sp. 10 originated from an intra-host duplication during the evolutionary history of *Pachychilon*, and that *Dactylogyrus* sp. 10 with *D. martinovici* originated from cospeciation during the divergence of *Pachychilon* species. Additionally, *D. rosickyi* is phylogenetically close to *Dactylogyrus* species from *Barbus* spp., which suggests a more recent host switch of parasites between these phylogenetically distant cyprinoid taxa. *Dactylogyrus rosickyi* was collected only from

P. pictum in the Aoos River (north-western Greece, a tributary of the Adriatic Sea), where the occurrence of *Barbus* species (*B. prespensis*) was also documented, and this *Dactylogyrus* species was not present on *P. pictum* in Lake Ohrid. *Dactylogyrus rosickyi* was originally described by Ergens (1970) from Lake Skadar. Both lakes are part of Ohrid-Drin-Skadar system. This system potentially represents the area within a range of *D. rosickyi* where the initial transfer between ancestral *Barbus* lineages and *Pachychilon* spp. took place.

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DATA AVAILABILITY STATEMENT

All new sequences of *Dactylogyrus* obtained during this study were deposited in NCBI GenBank under accession numbers MK434927–MK434965, MK455795 and MK455801. New sequences of cyprinoid species obtained during this study were deposited in NCBI GenBank under accession numbers MK482020–MK482050 (Table S1). Appropriate accession numbers according to *Dactylogyrus* species and specific genes coding rRNA regions are presented in Table S2. Since whole fish specimens were completely processed during parasitological dissection, additional specimens of each analysed host species were collected from the same locality and fish vouchers were deposited in the ichthyological collection of the National Museum in Prague (Czech Republic). Voucher specimens of the sequenced *Dactylogyrus* species (excluding undescribed species) are deposited in the Finnish Museum of Natural History in Helsinki.

ORCID

Michal Benovics https://orcid.org/0000-0002-4358-9332

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 List of cyprinoid species including localities of their collection and accession numbers for complete cytochrome *b* sequences deposited in GenBank.

Table S2 List of all collected *Dactylogyrus* species and their cyprinoid hosts.

Data S1 Alignment of cyprinoid DNA sequences used for phylogenetic analyses.

Data S2 Alignment of *Dactylogyrus* DNA sequences used for phylogenetic analyses.

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PAPER V

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

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Author for correspondence:
Michal Benovics,
E-mail: benovics@mail.muni.cz

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

Michal Benovics[1], Yves Desdevises[2], Radek Šanda[3], Jasna Vukić[4], Mathilde Scheifler[2], Ignacio Doadrio[5], Carla Sousa-Santos[6] and Andrea Šimková[1]

[1]Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic; [2]Sorbonne Université, CNRS, Biologie Intégrative des Organismes Marins, BIOM, Observatoire Océanologique, F-66650, Banyuls/Mer, France; [3]National Museum, Václavské Náměstí 68, 115 79 Prague, Czech Republic; [4]Department of Ecology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague, Czech Republic; [5]Biodiversity and Evolutionary Group, Museo Nacional de Ciencias Naturales-CSIC, C/José Gutiérrez Abascal 2, 28006, Madrid, Spain and [6]MARE – Marine and Environmental Sciences Centre, ISPA – Instituto Universitário, Rua Jardin do Tabaco 34, 1149-041, Lisbon, Portugal

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

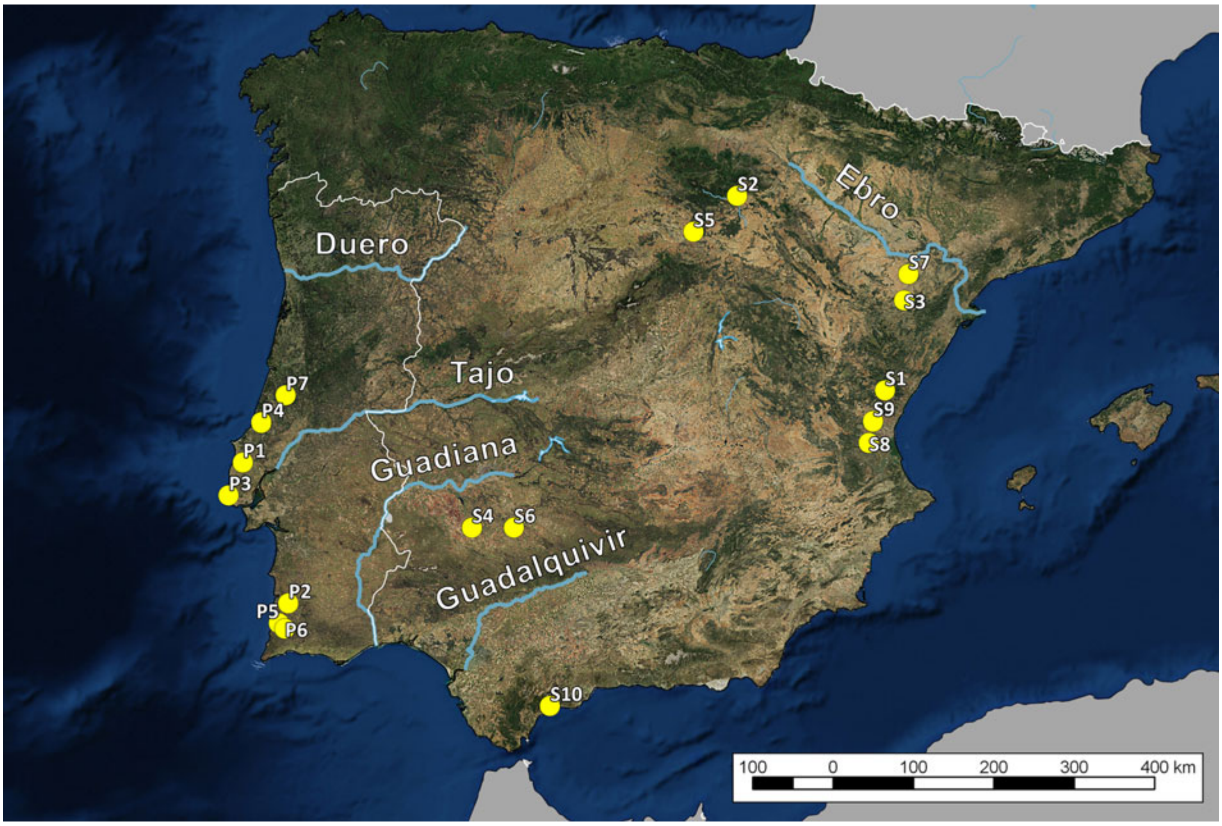

Fig. 1. Map of collection localities in the Iberian Peninsula. Collection localities are marked as yellow circles. The greatest Iberian rivers are highlighted in blue. The same codes for localities are used in Table 1 as locality IDs.

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 bocagei* exhibited the widest host range across the Iberian Peninsula, parasitizing four *Luciobarbus* species. Minor genetic variation was observed between *D. bocagei* 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. bocagei*	*L. bocagei*	P3	X										
2	*D. bocagei*	*L. comizo*	S6	–	X									
3	*D. bocagei*	*L. graellsii*	S7	0.001	0.001	X								
4	*D. bocagei*	*L. guiraonis*	S8	0.001	0.001	0.002	X							
5	*D. bocagei*	*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

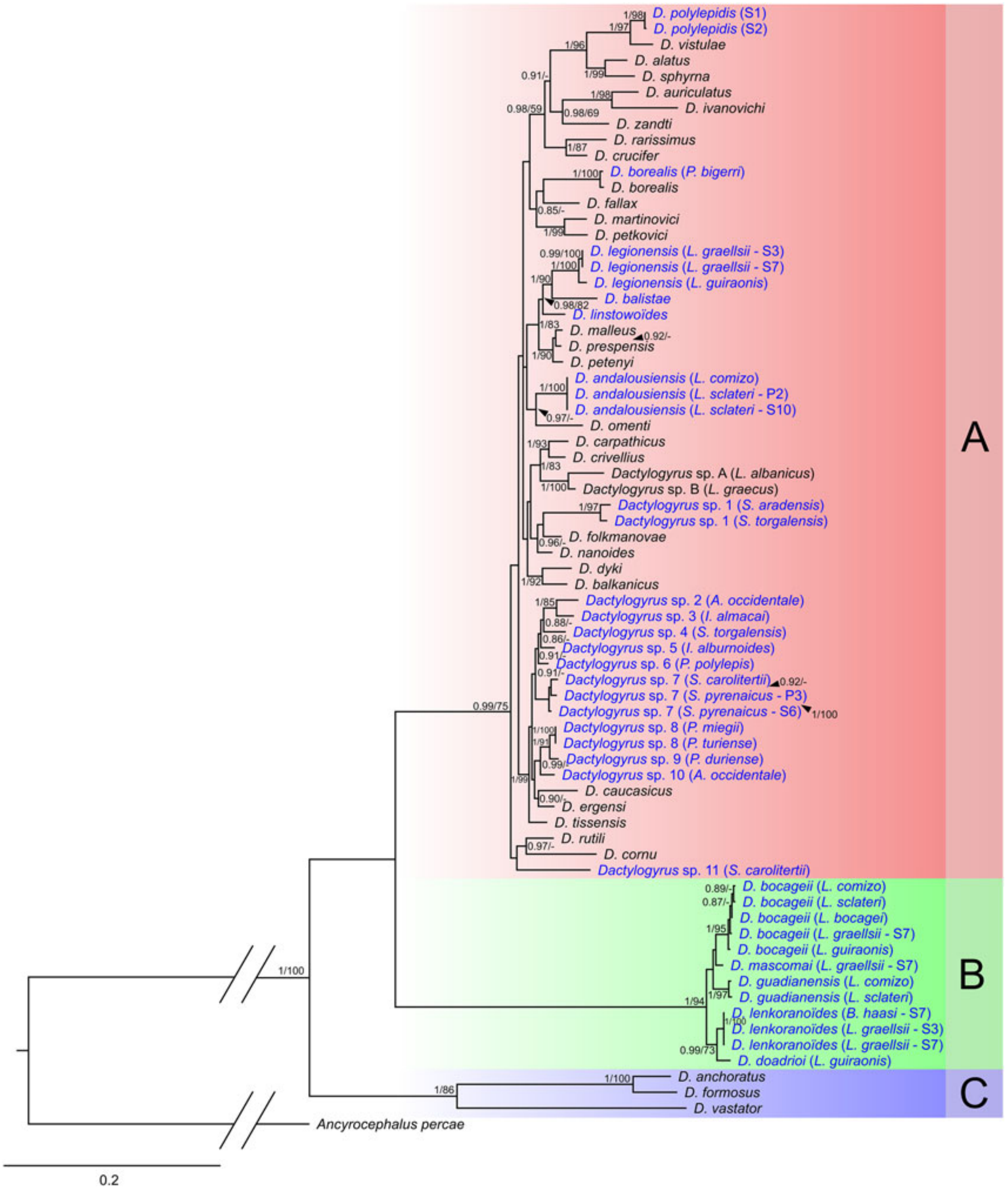

Fig. 2. Phylogenetic tree of 70 *Dactylogyrus* haplotypes reconstructed by Bayesian inference (BI). The tree is based on combined parts of genes coding 18S and 28S rRNA, and the complete ITS1 region. Values between branches indicate posterior probabilities from BI and bootstrap values from ML analysis. 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. bocageii*	*L. bocageii*	P3	X										
2	*D. bocageii*	*L. comizo*	S6	–	X									
3	*D. bocageii*	*L. graellsii*	S7	–	–	X								
4	*D. bocageii*	*L. guiraonis*	S8	–	–	–	X							
5	*D. bocageii*	*L. sclateri*	P2	–	–	–	–	X						
6	*D. 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.*,

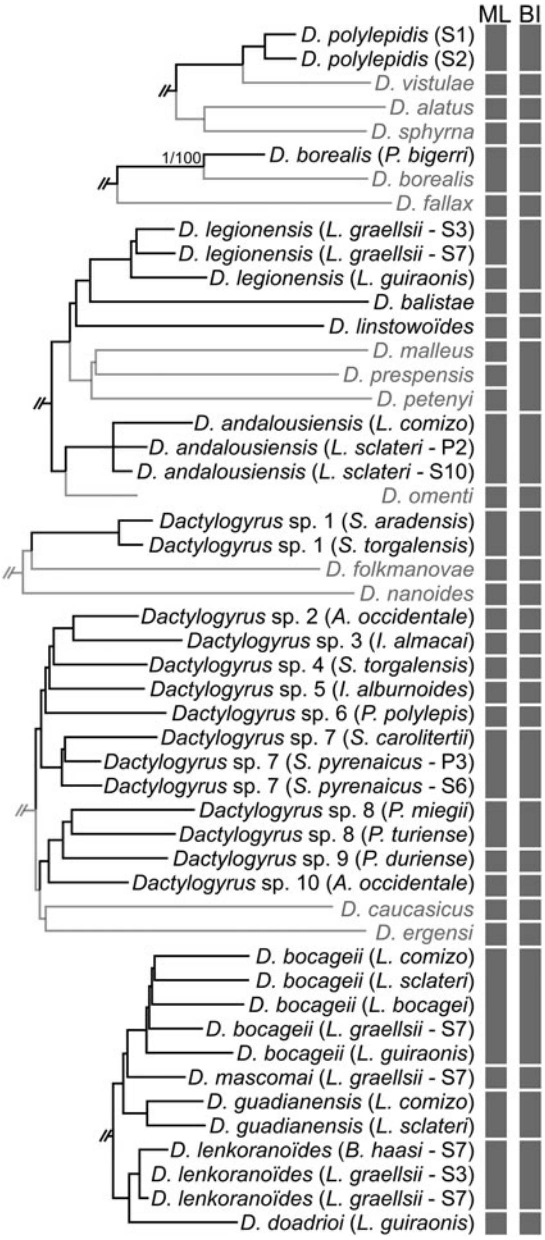

Fig. 3. Results of species bPTP delimitation analysis applied to clades comprising endemic *Dactylogyrus*. Brackets at the terminal branches indicate different species, as suggested by BI and ML analyses.

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

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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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