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Four new species of *Dactylogyrus* Diesing, 1850 (Monogenea: Dactylogyridae) parasitising the gills of northern Moroccan *Luciobarbus* Heckel (Cyprinidae): morphological and molecular characterisation

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Abstract Four new species of *Dactylogyrus* Diesing, 1850 are described from the gills of three species of *Luciobarbus* Heckel collected from various hydrographical basins in northern Morocco: *Dactylogyrus scorpius* n. sp. from *Luciobarbus rifensis* Doadrio, Casal-Lopez & Yahyaoui; *D. benhoussai* n. sp. from *L. moulouyensis* Pellegrin; and *D. varius* n. sp. and *D. falsiphallus* n. sp. from *L. maghrebensis* Doadrio, Perea & Yahyaoui. The descriptions of the new species are confirmed by molecular data (partial 18S rDNA, ITS1, and partial 28S rDNA sequences). All four species belong to the group of *Dactylogyrus* species, possessing a cross-shaped ventral bar and a male copulatory organ composed of a loosely coiled copulatory tube and an accessory piece with a capsule-like base and recurved distal portion. Given the high shape variability of the haptoral anchors reported among specimens of *D. varius* n. sp., three morphological forms within this species (*D. varius* f. *vulgaris*, *D. varius* f. *magnus*, and *D. varius* f. *dromedarius*) are recognised. However, specimens belonging to *D. benhoussai* n. sp. and *D. varius* f. *vulgaris* were morphologically very similar and were discriminated with certainty, only when using molecular data.

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Introduction

The Cyprinidae is one of the most widespread and diverse families of freshwater fishes, consisting of more than 2,400 species (220 genera) and occurring naturally in almost all types of habitat on all continents except for Australia and South America (Nelson, 2006). Moroccan cyprinids are represented by four

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genera, of which *Luciobarbus* Heckel is the most diverse, with 15 (14 endemic) currently recognised species (Eschmeyer et al., 2016). This genus includes also highly endemic species from the Iberian and Greek Peninsulas that were supposed to be closely related to North African ones (Machordom et al., 1995; Zardoya & Doadrio, 1999; Machordom & Doadrio, 2001; Doadrio et al., 2002). However, Tsigenopoulos et al. (2003) showed that North African *Luciobarbus* are phylogenetically closely related to those from the Middle East and proposed that the Lago Mare phase of the Mediterranean Sea following the Messinian salinity crisis was responsible for the actual biogeographical distribution of species belonging to this genus. Two genera, *Carasobarbus* Karaman, whose members occur in both northwestern Africa and southwestern Asia (Borkenhagen & Krupp, 2013) and *Labeobarbus* Rüppel, which are distributed in Africa and the Middle East (Tsigenopoulos et al., 2003), are each represented by only two species in Morocco. The last Moroccan cyprinid genus *Pterocapoeta* Günther is monotypic and endemic (Vreven et al., 2016).

Cyprinids are known to harbour species of *Dactylogyrus* Diesing, 1850 (Dactylogyridae, Monogenea), one of the largest helminth genera, with more than 900 nominal species (Gibson et al., 1996). This number is explained by the high diversity of their cyprinid hosts and by their high host specificity, i.e. many *Dactylogyrus* species are specific to a single host species (i.e. strict host specificity) or to congeneric hosts (Šimková et al., 2006). To date, only 13 species of *Dactylogyrus* have been described from three genera of Moroccan cyprinids, i.e. *Carasobarbus*, *Labeobarbus,* and *Luciobarbus* (see El Gharbi et al., 1994). Among them, only seven species exhibit strict host specificity: *D. atlasensis* El Gharbi, Birgi & Lambert, 1994; *D. borjensis* El Gharbi, Birgi & Lambert, 1994; *D. draaensis* El Gharbi, Birgi & Lambert, 1994; *D. guirensis* El Gharbi, Birgi & Lambert, 1994; *D. reinii* El Gharbi, Birgi & Lambert, 1994; *D. volutus* El Gharbi, Birgi & Lambert, 1994; and *D. zatensis* El Gharbi, Birgi & Lambert, 1994. The six remaining species exhibit congeneric (stenoxenous) host specificity: *D. fimbriphallus* El Gharbi, Birgi & Lambert, 1994; *D. ksibii* El Gharbi, Birgi & Lambert, 1994; *D. ksibioides* El Gharbi, Birgi & Lambert, 1994; *D. kulindrii* El Gharbi, Birgi & Lambert, 1994; *D. marocanus* El Gharbi, Birgi & Lambert, 1994; and *D. oumiensis* El Gharbi, Birgi & Lambert, 1994 (see El Gharbi et al., 1994).

Recent investigations and new knowledge on the biological diversity of Moroccan cyprinids based on the application of molecular markers for phylogenetic reconstruction (Machordom et al., 1998; Machordom & Doadrio 2001; Casal-Lopez et al., 2015; Doadrio et al., 2016), and the need to extend the survey to the south of the Atlas Mountains (Drâa Valley) lead us to re-evaluate the monogenean fauna of these fish hosts. In the present study, four new species of *Dactylogyrus* are described from the gills of three species of *Luciobarbus* using a combination of morphological and molecular approaches.

Materials and methods

Collection and identification

The fish hosts *Luciobarbus maghrebensis* Doadrio, Perea & Yahyaoui; *L. moulouyensis* Pellegrin; and *L. rifensis* Doadrio, Casal-Lopez & Yahyaoui were sampled from three different hydrographical basins in Morocco, the Loukkos Basin, the Sebou Basin, and the Moulouya Basin (Fig. 1) by means of gill nets or electro-fishing. In addition, *Luciobarbus ksibi* Boulenger from the Ksob Basin (Fig. 1), was examined for the presence of *Dactylogyrus* specimens used for the comparative morphometric and molecular analyses. Fishes were transported live to the field laboratory, sacrificed by severing the spinal cord and dissected immediately. The methods used for parasite collection and preparation for taxonomic evaluation were as described in Musilová et al. (2009). Specimens of *Dactylogyrus*, fixed with a mixture of glycerine and ammonium picrate (GAP; Malmberg, 1957), were studied using an Olympus BX51 microscope equipped with phase contrast optics, and drawings were made with the aid of a video camera. Measurements of the sclerotised structures (the haptoral and reproductive hard parts) were taken using ImageJ software (available at: http://rsb.info.nih.gov/ij/), and are given in micrometres as the range, followed by the mean in parentheses. The scheme of measurement for the hard structures is shown in Fig. 2. The haptoral terminology follows Řehulková et al. (2013). The male copulatory organ is abbreviated below to MCO. The numbering of hooks is that proposed by Mizelle (1936). The type-specimens were deposited in the collection of the Muséum National d'Histoire Naturelle, Paris (MNHN). Note that the authors of the new

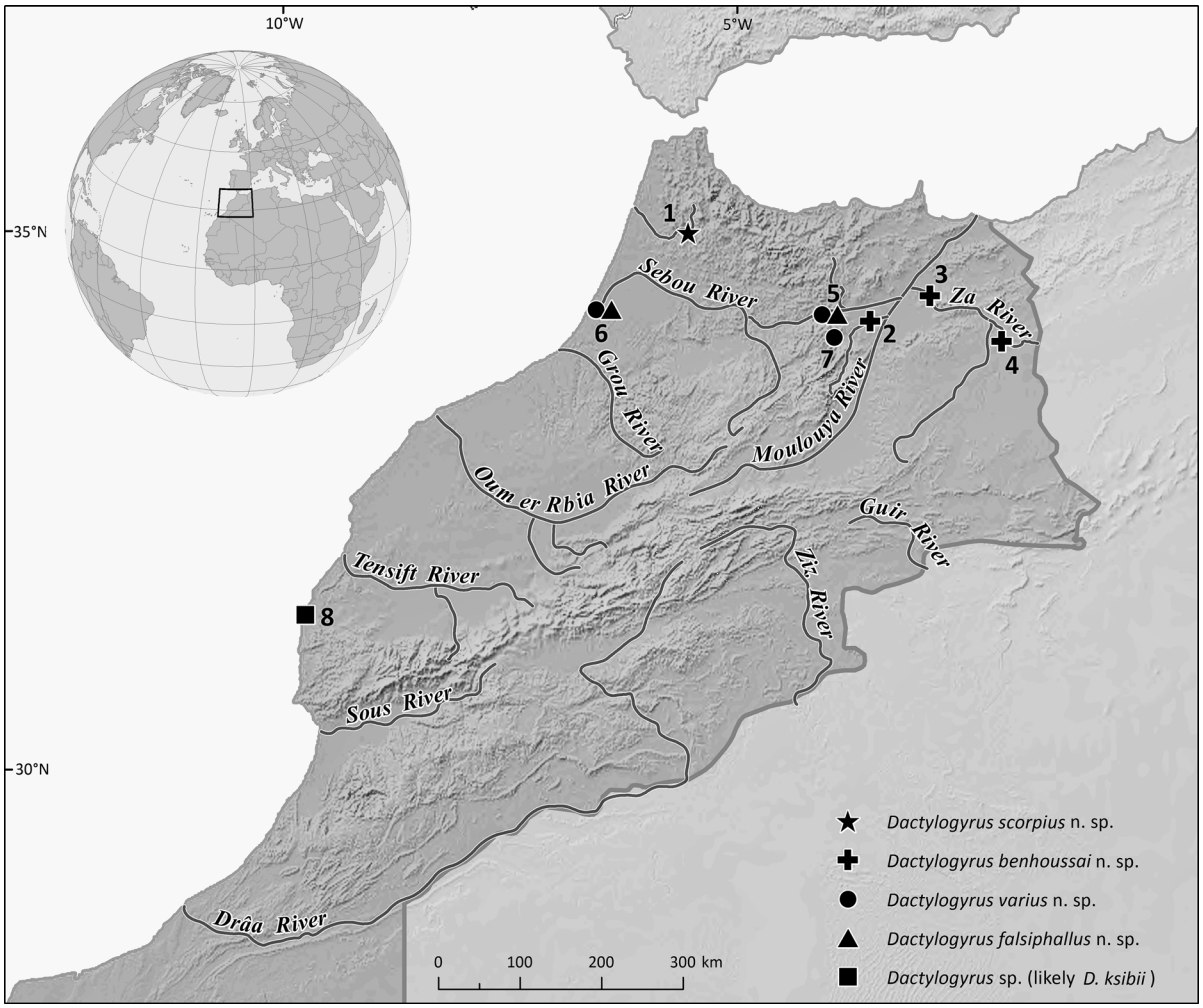

Fig. 1 Sampling localities of *Luciobarbus* spp. populations in northern Morocco: 1, River Loukkos; 2, River Melloulou; 3, River Za; 4, River Charef; 5, River Lahdar; 6, River Sebou; 7, River Saghor; 8, River Ksob

taxa are different from the authors of this paper; see Article 50.1 and Recommendation 50A of the International Code of Zoological Nomenclature.

DNA isolation and PCR amplification

Parasite specimens collected for DNA analyses were bisected using fine needles under a dissecting microscope. Subsequently, one half of the body (in most cases, the anterior part containing the MCO) was fixed in 96% ethanol for later molecular analyses, i.e. the sequencing of selected regions of rRNA genes; the other body half was completely flattened under coverslip pressure and fixed with GAP for species identification. DNA was extracted using DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and then concentrated to a final volume of 80 µl. The amplification of partial 18S ribosomal DNA and the entire first internal transcribed spacer (ITS1) was performed by using the forward primer S1 (5′-ATT CCG ATA ACG AAC GAG ACT-3′) and the reverse primer IR8 (5′-GCT AGC TGC GTT CTT CAT CGA-3′) (Šimková et al., 2003). The amplification of partial 28S ribosomal DNA was performed using the forward primer C1 (5′-ACC CGC TGA ATT TAA GCA T-3′) and the reverse primer D2 (5-TGG TCC GTG TTT CAA GAC-3′) (Hassouna et al., 1984). For the combined partial 18S rDNA and ITS1, PCR was carried out in a total volume of 30 µl containing 5 µl of DNA extract, 1× PCR buffer, 0.1 mg/ml BSA, 1.5 mM $MgCl_2$, 200 µM

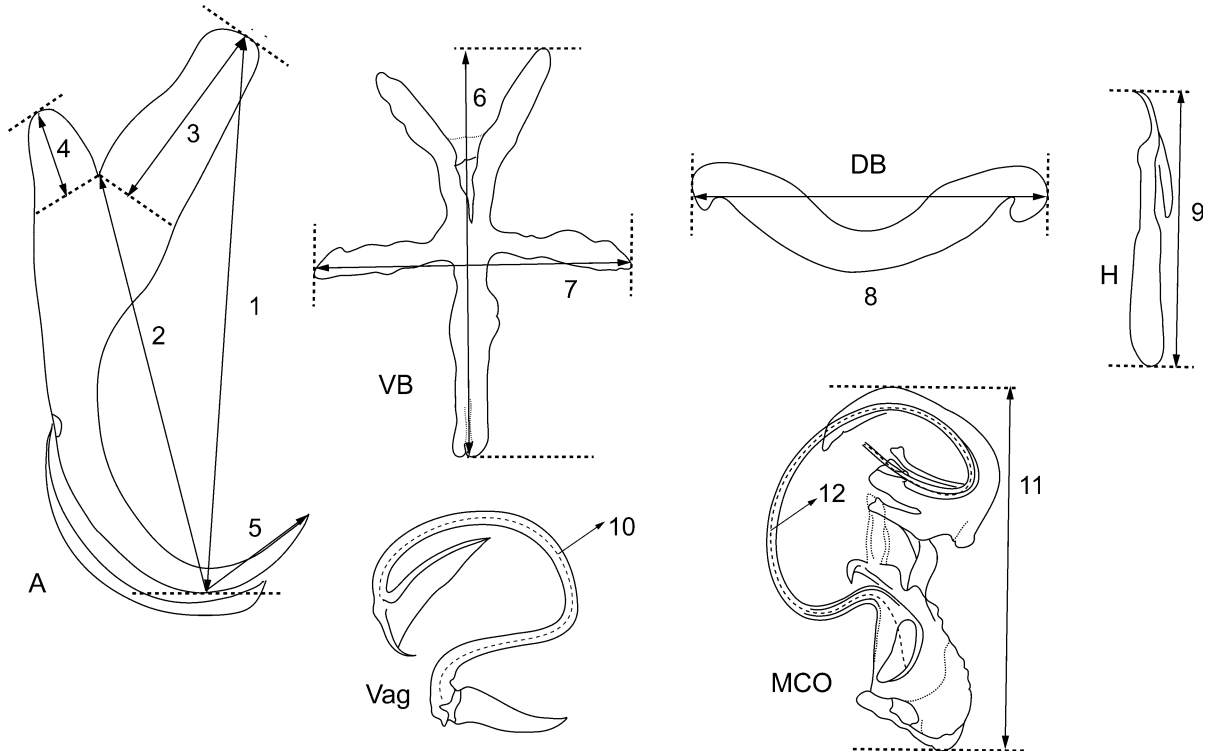

Fig. 2 Scheme for the measurements of the sclerotised structures of haptor and reproductive organs of *Dactylogyrus* spp. *Key*: A, anchor (1, total length; 2, length to notch; 3, inner root length; 4, outer root length; 5, point length); VB, ventral bar (6, total length; 7, total width); DB, dorsal bar (8, total width); H, hook (9, total length); Vag, vagina (10, length); MCO, male copulatory organ (11, total length; 12, tube length)

dNTPs, 0.5 µM of each PCR primer, and 1.5 U *Taq* DNA polymerase. PCR amplification was achieved with the following steps: 2 min at 94°C followed by 39 cycles of 1 min at 94°C, 1 min at 53°C and 1 min 30 s at 72°C, and a final extension of 10 min at 72°C. For partial 28S rDNA, PCR was performed in a total volume of 30 µl containing 5 µl of DNA extract, 1× buffer, 0.1 mg/ml BSA, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each PCR primer, and 1.5 U *Taq* DNA polymerase; PCR amplification was achieved with the following steps: 2 min at 94°C followed by 39 cycles of 20 s at 94°C, 30 s at 58°C, and 1 min 30 s at 72°C, and a final extension of 10 min at 72°C. PCR products were examined on 1% agarose TBE gel, stained with Good View (SBS Genetech, Bratislava, Slovakia), visualised under UV light, and documented using the GBox F3 Bio Imaging System (Syngene, Cambridge, UK). PCR products were purified using Exo SAP-IT kit (Affymetrix, Santa Clara, CA, USA). Sequencing was carried out using the same primers as for PCR on an ABI 3130 Genetic Analyzer (Applied Biosystems) using a Big Dye Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems). Sequences were analysed using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA) and deposited in the GenBank database under accession numbers KX553860–KX553864 and KX578023–KX578027. The alignment of the obtained sequences for each data set was performed using Clustal W multiple alignments (Thompson et al., 1994), incorporated in MEGA v. 6 (Tamura et al., 2013). Uncorrected p-distances between the *Dactylogyrus* species described in this study were calculated using MEGA V.6 software.

Results

Four species of *Dactylogyrus* were found on three host species of *Luciobarbus* (*L. maghrebensis*, *L. moulouyensis* and *L. rifensis*) collected from three different hydrographical basins, namely the Loukkos

Basin, the Sebou Basin, and the Mouloya Basin. All *Dactylogyrus* spp. belonged morphologically to the 'carpathicus' group, as defined by El Gharbi et al. (1994), i.e. a group of *Dactylogyrus* species having a cross-shaped ventral bar and MCO with a capsule-like base. The basic structure of the MCO of four of the previously described species of the 'carpathicus' group, i.e. *Dactylogyrus atlasensis*, *D. borjensis*, *D. ksibii* and *D. ksibioides*, parasitising Moroccan *Luciobarbus* spp., suggests a relationship with the specimens of *Dactylogyrus* studied here. However, the drawings of the sclerotised structures provided by El Gharbi et al. (1994), particularly those of the MCO, lack sufficient detail for specific differentiation. Unfortunately, these authors failed to deposit type-material of the *Dactylogyrus* spp. described in a museum (J.-L. Justine, personal communication). Thus, comparison of our specimens of *Dactylogyrus* spp. with type-specimens of the previously described species mentioned above was not possible.

Order Dactylogyridea Bychowsky, 1937
Family Dactylogyridae Bychowsky, 1933
Genus *Dactylogyrus* Diesing, 1850

Dactylogyrus scorpius Rahmouni, Řehulková & Šimková n. sp.

Type-host: *Luciobarbus rifensis* Doadrio, Casal-Lopez & Yahyaoui (Cyprinidae), Rifian barbel.
Type-locality: River Loukkos (34°54′57.2″N, 5°32′17.2″W), Morocco.
Type-material: Holotype (MNHN HEL567) and 2 paratypes (MNHN HEL568).
Site on host: Gill lamellae.
Representative DNA sequences: GenBank accession numbers: KX553860 (28S rDNA), KX578023 (18S rDNA and ITS1).
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, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *D. scorpius* n. sp. is urn:lsid:zoobank.org:act:0ABBC656-BA09-4561-AC71-9ED D7F896D0D.
Etymology: The specific epithet reflects the scorpion's curved tail appearance of the accessory piece.

Description (Figs. 3, 7A)

[Based on 21 specimens.] Body length 400–711 (517); greatest width 60–100 (78) at level of ovary. Haptor with 1 pair of anchors (dorsal): total length 40–54 (48); length to notch 30–40 (37); inner root 15–20 (18) long; outer root 5–9 (7) long; shaft curved; point extending just past level of tip of inner root, 8–10 (10) long. One pair of needles located near hooks of pair V. Dorsal bar broadly V-shaped, with narrowed medial part and subterminal notches, 26–32 (30) long. Ventral bar cross-shaped, with 5 arms, 35–43 (39) long, 25–35 (31) wide. Hooks 7 pairs, similar in shape; each with delicate point, depressed truncate thumb, shank comprised of 2 subunits (proximal subunit expanded); hook filament (HF) loop extending to near level of termination of shank inflation; hook lengths: pair I: 21–27 (25); pair II: 20–26 (24); pair III: 25–30 (28); pair IV: 24–29 (27); pair V: 22–28 (26); pair VI: 24–29 (27); pair VII: 25–28 (27). MCO complex, comprising basally articulated copulatory tube and accessory piece of total length 24–29 (26). Copulatory tube a loose coil following S-shaped path with less than 1 complete terminal ring, distally narrowing to delicate (poorly defined) termination, 43–46 (45) long. Accessory piece proximally enclosing base of copulatory tube to form frill-belted capsule-like structure; distal portion recurved, following half of medial part of copulatory tube just before its distal recurving; medial portion with 3 processes: primary process ridge-like, articulated to capsule by lightly sclerotised ligament; secondary process more robust, terminally grooved, serving as guide for distal part of copulatory tube; tertiary process like crescent-shaped paddle, terminally closely associated with secondary process. Vagina a slightly sclerotised wavy tube, with disc-shaped opening supported by usually three sclerotised finger-like rays, 30–41 (36) long.

Molecular characterisation

The sequence of partial 28S rDNA of *D. scorpius* n. sp. was 792 bp long. The sequence of partial 18S rDNA, entire ITS1 region and partial 5.8S rDNA of *D. scorpius* n. sp. was 992 bp long, of which 492 bp corresponded to 18S rDNA, 488 bp corresponded to ITS1 region, and 12 bp corresponded to 5.8S rDNA. Three specimens of *Dactylogyrus scorpius* n. sp. from River Loukkous were sequenced, and no intraspecific

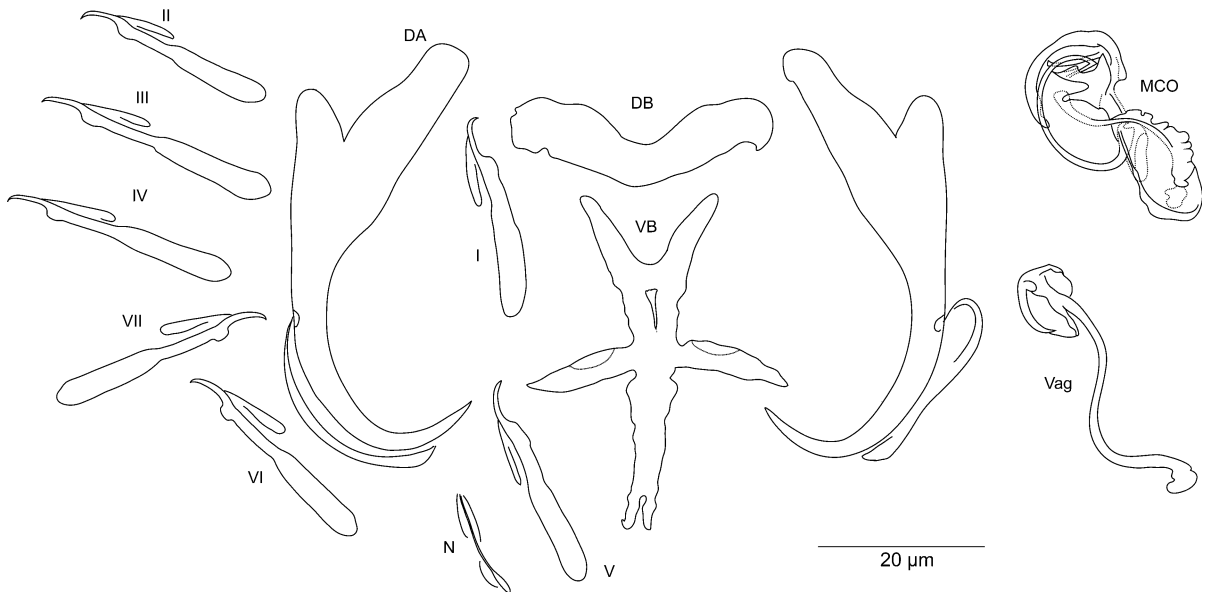

Fig. 3 Sclerotised structures of *Dactylogyrus scorpius* n. sp. ex *Luciobarbus rabatensis*. *Abbreviations*: DA, dorsal anchor; DB, dorsal bar; VB, ventral bar; N, needle; I-VII, hooks (pairs I-V ventral; pairs VI, VII dorsal); MCO, male copulatory organ; Vag, vagina

variability was found between these specimens. Pairwise distances between *D. scorpius* n. sp. and the three other *Dactylogyrus* spp. analysed using molecular markers are shown in Table 1. Using the combined sequences of the different molecular markers, pairwise comparisons between the *Dactylogyrus* species described in this study showed that *D. scorpius* n. sp. is the species with the highest similarity to *D. benhoussai* n. sp. (1.9% of molecular divergence calculated using p-distances for the combined sequences).

Table 1 Pairwise distances calculated using number of different nucleotides (above diagonal) and uncorrected p-distances (below diagonal)

	D. scorpius	*D. falsiphallus*	*D. benhoussai*	*D. varius*
28S rDNA				
D. scorpius	–	16	9	12
D. falsiphallus	0.020	–	16	19
D. benhoussai	0.011	0.020	–	3
D. varius	0.015	0.024	0.004	–
18S rDNA				
D. scorpius	–	2	3	2
D. falsiphallus	0.004	–	3	2
D. benhoussai	0.006	0.006	–	1
D. varius	0.004	0.004	0.002	–
ITS1				
D. scorpius	–	35	21	23
D. falsiphallus	0.072	–	26	29
D. benhoussai	0.043	0.053	–	14
D. varius	0.047	0.060	0.029	–

Remarks

Dactylogyrus scorpius n. sp. most closely resembles *Dactylogyrus ksibii* described on the gills of *Barbus* (*Barbus*) *ksibi* Boulenger (syn. *Luciobarbus ksibi*), *B.* (*B.*) *setivimensis* Valenciennes (syn. *Luciobarbus setivimensis*) and *B.* (*B.*) *magniatlantis* Pellegrin (syn. *Luciobarbus magniatlantis*) in Morocco by El Gharbi et al. (1994). On the basis of the original drawings, both species possess morphologically comparable haptoral structures (anchors with well-developed roots, slightly bent shaft and short point; saddle-shaped dorsal bar; cross-shaped ventral bar with five arms; hooks of similar shape and size) and MCOs (copulatory tube with capsule-like base; accessory piece with recurved distal portion and medial portion with three poorly defined processes). However, El Gharbi et al. (1994) indicated that the MCO was highly morphologically variable among specimens of *D. ksibii*, as illustrated in their figures 16 b-e, but did not specify which one was typical. In addition, these authors also mentioned some

morphological variability in the sclerotised haptoral parts of specimens of *D. ksibii* collected from different hosts and localities. Thus, it is possible that at least two different species of *Dactylogyrus* were included in the original description of *D. ksibii*. Because of the absence of type-specimens and the inadequacy of the original description, *D. ksibii* should be redescribed together with the designation of a neotype. Also, the parasitological examination of *L. ksibi*, *L. setivimensis* and *L. magniatlantis*, the potential hosts for previously described *D. ksibii* from different localities in Morocco, will be necessary. Given the above and the fact that the type-host and locality for *D. ksibii* is *L. ksibi* and the Ksaba River (Essaouira), respectively, we sequenced partial 28S rDNA and the combined 18S rDNA and ITS1 from specimens of *Dactylogyrus* (likely *D. ksibii* based on morphological comparison with the original drawings) collected by us from the type-host species and locality (31°27′50.7″N, 9°45′25.3″W; i.e. locality 8 in Fig. 1). Our molecular analysis revealed molecular divergence between *D. scorpius* n. sp. and these *Dactylogyrus* specimens (likely *D. ksibii*) (2% for combined sequence using all molecular markers, 1.5% for partial 28S rDNA, 0.6% for partial 18S rDNA and 4.3% for ITS1 region). The sequences of *Dactylogyrus* specimens (likely *D. ksibii*) were deposited as *Dactylogyrus* sp. from *Luciobarbus ksibi* in the GenBank under accession numbers KX553864 (28S rDNA), KX578027 (18S rDNA and ITS1).

Dactylogyrus benhoussai Rahmouni, Řehulková & Šimková n. sp.

Type-host: *Luciobarbus moulouyensis* Pellegrin (Cyprinidae), Moulouyan barb.
Type-locality: River Melloulou (34°10′51.7″N, 3°31′59.6″W), Morocco.
Other localities: River Za (34°24′38.9″N, 2°52′29.1″W), River Charef (33°59′50.3″N, 2°05′07.3″W), Morocco.
Type-material: Holotype (MNHN HEL569) and 3 paratypes (MNHN HEL570).
Site on host: Gill lamellae.
Representative DNA sequences: GenBank accession numbers: KX553862 (28S rDNA), KX578025 (18S rDNA and ITS1).
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, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *D. benhoussai* n. sp. is urn:lsid:zoobank.org:act:5B73B93E-D856-4DF8-A922-6CE5AAFBD0EB.
Etymology: This species is named in honour of Professor Abdelaziz Benhoussa, Mohamed V University of Rabat, who helped us in the collection of fish specimens.

Description (Figs. 4, 7B)

[Based on 28 specimens.] Body length 404–762 (535); greatest width 85–123 (102) at level of ovary. Haptor with 1 pair of anchors (dorsal): total length 35–45 (39); length to notch 27–34 (31); inner root 11–17 (15) long; outer root 4–7 (5) long; shaft curved; point extending just past level of tip of inner root, 7–10 (9) long. One pair of needles located near hooks of pair V. Dorsal bar broadly V-shaped, with slightly narrowed medial part and subterminal notches, 25–30 (28) long. Ventral bar cross-shaped, with 5 arms, 27–31 (29) long, 21–26 (24) wide. Hooks 7 pairs, similar in shape; each with delicate point, depressed truncate thumb, shank comprised of 2 subunits (proximal subunit expanded); HF loop extending to near level of termination of shank inflation; hook lengths: pair I: 20–24 (22); pair II: 20–25 (23); pair III: 23–30 (26); pair IV: 21–25 (24); pair V: 21–25 (23); pair VI: 21–26 (24); pair VII: 24–29 (26). MCO complex composed of basally articulated copulatory tube and accessory piece of total length 26–30 (28). Copulatory tube a loose coil following S-shaped path with less than 1 complete terminal ring, narrowing to delicate termination, 66–69 (67) long. Accessory piece proximally enclosing base of copulatory tube to form frill belted capsule-like structure; distal portion recurved, following half of the medial part of copulatory tube just before its distal recurving; medial portion with 3 processes: primary process finger-like, articulated to the capsule by lightly sclerotised ligament; secondary process terminally grooved, serving as guide for distal part of copulatory tube; tertiary process like crescent-shaped paddle, usually closely associated with secondary process in its terminal part. Vagina a lightly sclerotised wavy tube, with disc-shaped opening supported by sclerotised finger-like rays, 45–56 (49) long.

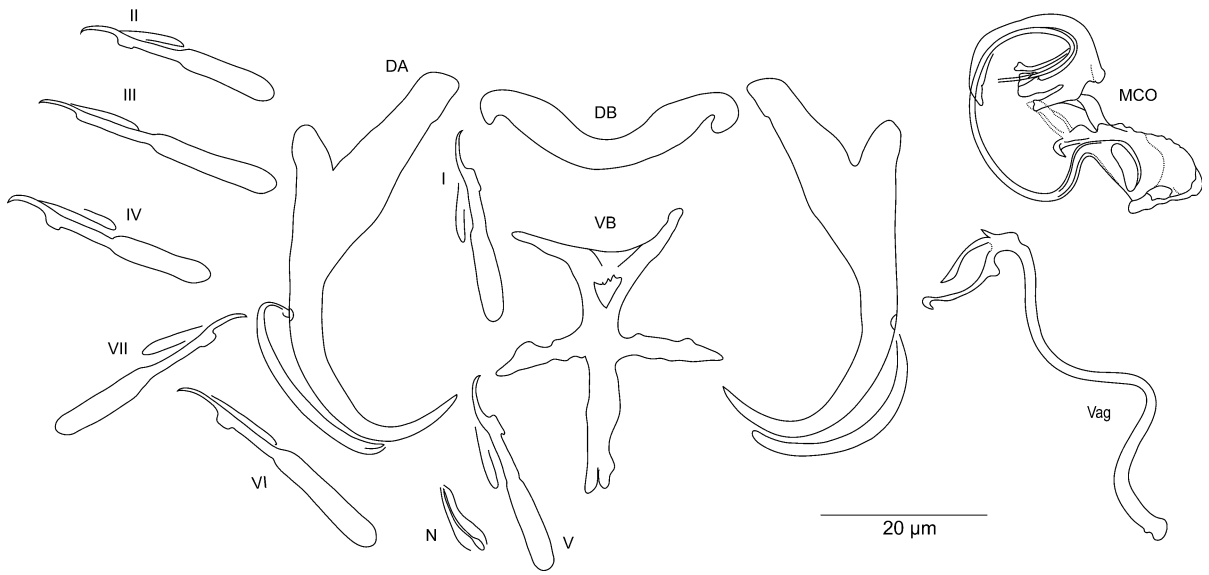

Fig. 4 Sclerotised structures of *Dactylogyrus benhoussai* n. sp. ex *Luciobarbus moulouyensis*. *Abbreviations*: DA, dorsal anchor; DB, dorsal bar; VB, ventral bar; N, needle; I-VII, hooks (pairs I-V ventral; pairs VI, VII dorsal); MCO, male copulatory organ; Vag, vagina

Molecular characterisation

The sequence of partial 28S rDNA of *D. benhoussai* n. sp. was 792 bp long. The sequence of partial 18S rDNA, entire ITS1 region and partial 5.8S rDNA of *D. benhoussai* n. sp. was 992 bp long, of which 492 bp corresponded to the 18S rDNA, 488 bp corresponded to the ITS1 region, and 12 bp corresponded to the 5.8S rDNA.Three specimens of *Dactylogyrus benhoussai* n. sp. from two different rivers (River Melloulou and River Za) were sequenced, and no intraspecific variability between these specimens was found. Pairwise distances between *D. benhoussai* n. sp. and the three other *Dactylogyrus* spp. analysed using molecular markers are shown in Table 1. Using the combined sequences of the different molecular markers, pairwise comparisons between the *Dactylogyrus* species described in this study showed *D. benhoussai* n. sp. and *D. varius* n. sp. to exhibit the highest molecular similarity (1% of molecular divergence calculated using p-distances).

Remarks

This new species is similar to *D. scorpius* n. sp., as shown by the comparative morphology of the haptoral parts and MCOs. *Dactylogyrus benhoussai* n. sp. is separated from this species by having (i) a longer vagina (49 *vs* 36 µm), (ii) a more robust MCO, and, *vice versa*, smaller haptoral structures, and (iii) a MCO with well-developed processes arising from medial part of the accessory piece (the medial processes of the accessory piece are more delicate in *D. scorpius*). Molecular analysis based on sequences of partial 28S rDNA and combined sequences of partial 18S rDNA and the entire ITS1 region also support the validity of both species.

Dactylogyrus varius Rahmouni, Řehulková & Šimková n. sp.

Type-host: *Luciobarbus maghrebensis* Doadrio, Perea & Yahyaoui (Cyprinidae), Maghreb barbel.
Type-locality: River Lahdar (34°14′32.7″N, 4°03′53.9″W), Morocco.
Other localities: River Sebou (34°17′14.2″N, 6°33′14.088″W), River Saghor (34°2′4.029″N, 3°55′45.584″W).
Type-material: Holotype (MNHN HEL571) and 5 paratypes (MNHN HEL572–MNHN HEL574).
Site on host: Gill lamellae.
Representative DNA sequences: GenBank accession numbers: KX553863 (28S rDNA), KX578026 (18S rDNA and ITS).

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, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *D. varius* n. sp. is urn:lsid:zoobank.org:act:D92D00C9-3F8A-404D-B652-323F29FF9296.

Etymology: The specific name is from Latin (*varius* = diverse, various) and refers to the shape variability of anchors.

Description (Figs. 5, 7C, 8)

[Based on 42 specimens.] Body length 322–795 (575); greatest width 66–190 (112) at level of ovary. Haptor with 1 pair of anchors (dorsal): total length 40–60 (48); length to notch 32–50 (38); inner root 14–24 (18) long; outer root 4–8 (6) long; shaft curved; point extending just past level of tip of inner root, 8–11 (10) long. One pair of needles located near hooks of pair V. Dorsal bar broadly V-shaped, with narrowed medial part and subterminal notches, 26–37 (32) long. Ventral bar cross-shaped, with 5 arms, 29–44 (36) long, 23–30 (27) wide. Hooks 7 pairs, similar in shape; each with delicate point, depressed truncate thumb, shank comprised of 2 subunits (proximal subunit expanded); HF loop extending near to level of termination of shank inflation; hook lengths: pair I: 22–28 (25); pair II: 20–30 (24); pair III: 23–33 (28); pair IV: 25–33 (23); pair V: 24–34 (28); pair VI: 26–34 (29); pair VII: 23–30 (26). MCO complex, composed of basally articulated copulatory tube and accessory piece of total length 23–31 (27). Copulatory tube a loose coil following S-shaped path with about 1 terminal ring, narrowing to delicate termination, 58–69 (65) long. Accessory piece proximally enclosing base of copulatory tube to form frill-belted capsule-like structure; distal portion recurved, following the half of medial part of copulatory tube just before its distal recurving; medial portion with three processes: primary process ridge-like, articulated to the capsule by lightly sclerotised ligament; secondary process more robust, terminally grooved, serving as a guide for distal part of copulatory tube; tertiary process like a crescent-shaped paddle, terminally closely associated with secondary process. Vagina a lightly sclerotised wavy tube, with disc-shaped opening supported by usually 2 sclerotised finger-like rays, 40–52 (45) long.

Molecular characterisation

The sequence of 28S rDNA was 792 bp long. The sequence of the partial 18S rDNA, entire ITS1 region and partial 5.8S rDNA of *D. varius* n. sp. was 992 bp long, of which 492 bp corresponded to the 18 rDNA, 488 bp corresponded to the ITS1 region, and 12 bp corresponded to the 5.8S rDNA. Twenty-four specimens of *Dactylogyrus varius*, from three different rivers (Lahdar, Sebou and Saghor) were sequenced and no nucleotide variability between the three different forms of *D. varius* n. sp. was found. Pairwise distances between *D. varius* n. sp. and three other congeneric species analysed using molecular markers are shown in Table 1. Using the combined sequences of the different molecular markers, pairwise comparisons between the *Dactylogyrus* spp. described in this study showed *D. varius* n. sp. and *D. benhoussai* n. sp. to exhibit the highest molecular similarity (1% of molecular divergence calculated using p-distances).

Remarks

Dactylogyrus varius n. sp. exhibits a great deal of variation in the shape (Figs. 5, 8) and size (Table 2) of its haptoral anchors, although the morphology of the other sclerotised haptoral parts is relatively stable. In individual specimens, the size of anchors ranges between 40–60 μm and shape variability in the inner roots of the anchors is so high that it appears to be sufficient for species delineation. However, the morphology of the MCO and that of the vagina is identical in all specimens examined. Furthermore, we found molecular evidence that, despite morphological divergences in the shape of the haptoral anchors, all specimens examined represent a single species. Basically, three morphological forms of *D. varius* n. sp. were recognised: *D. varius* f. *vulgaris*, *D. varius* f. *magnus*, and *D. varius* f. *dromedarius*. Figures 5 and 8 and Table 2 present the differentiation of the three forms: the inner root of the anchor in *D. varius* f. *vulgaris* is slightly expanded basally; in *D. varius* f. *magnus* it is evenly expanded along its inner side except for the terminal portion; and in *D. varius* f. *dromedarius* it is sharply expanded to form a conspicuous medial hump on its inner side.

The structure of the MCO suggests a close relationship between *D. varius* n. sp. and *D. benhoussai* n. sp., a species parasitising *L. moulouyensis*.

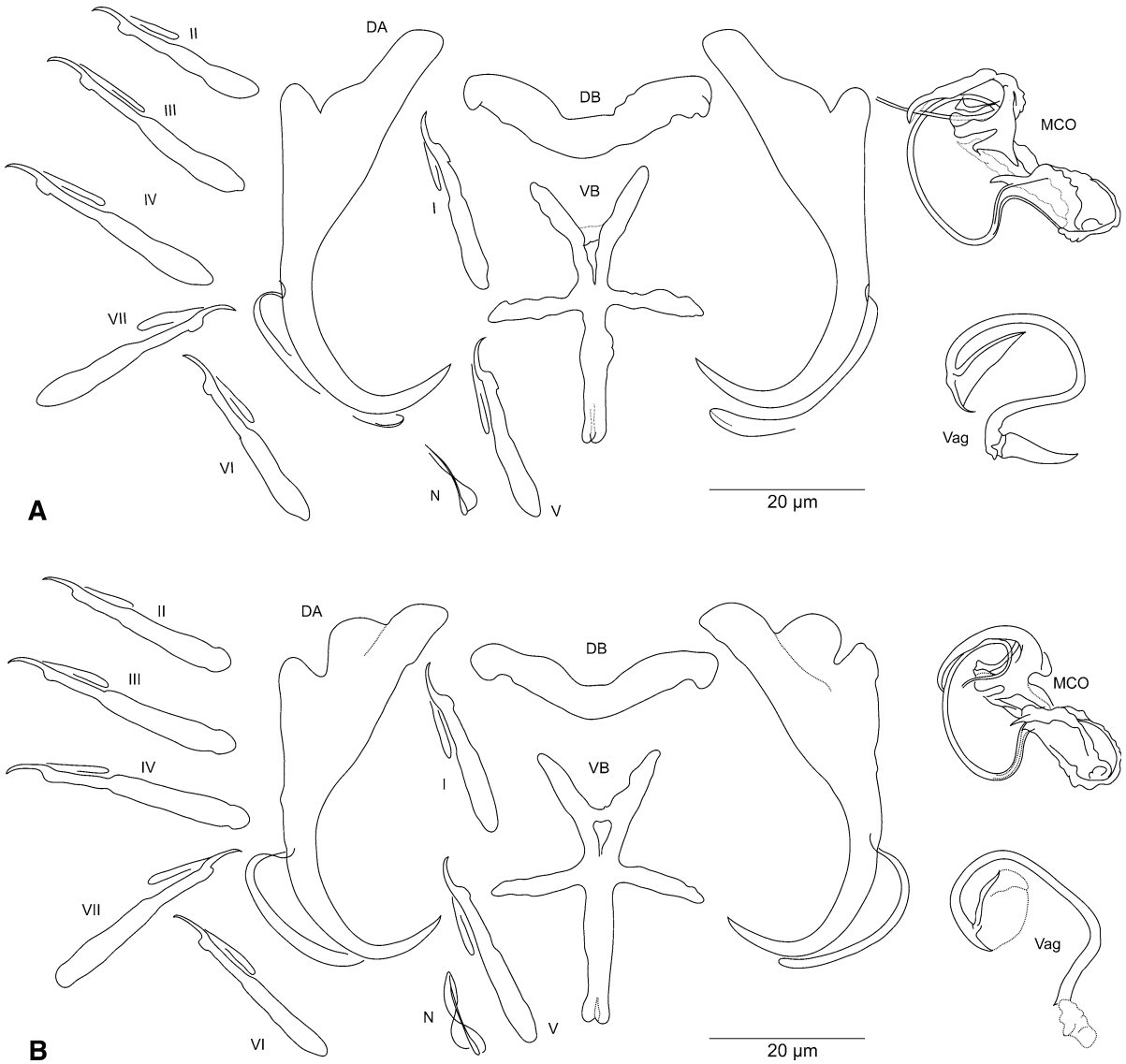

Fig. 5 Sclerotised structures of *Dactylogyrus varius* n. sp. ex *Luciobarbus maghrebensis.* A, forma *vulgaris*; B, forma *dromedarius*. *Abbreviations*: DA, dorsal anchor; DB, dorsal bar; VB, ventral bar; N, needle; I-VII, hooks (pairs I-V ventral; pairs VI, VII dorsal); MCO, male copulatory organ; Vag, vagina

Dactylogyrus varius f. *vulgaris* could even be confused with *D. benhoussai* n. sp. not only by having a morphologically similar (cryptic) MCO, but also because differences in the haptoral sclerites of the two taxa are minimal and could fall within the "expected" intraspecific variation among dactylogyrids. However, detailed comparison of the morphology of the anchors, accessory piece, and vagina are apparently the best means of separating specimens and, together with sequence-based species delimitation (see above), provide sufficient evidence that the two species are valid. In *D. varius* f. *vulgaris*, the anchors are slightly larger (mean 48 *vs* 39 µm; range 41–56 *vs* 35–45 µm) and possess inner roots with a wider (bulgy) base; in addition, the medial processes and distal part of the accessory piece are more delicate than the respective structures in *D. benhoussai* n. sp.

The other two forms of *D. varius* n. sp., i.e. forma *magnus* and forma *dromedarius*, are easily

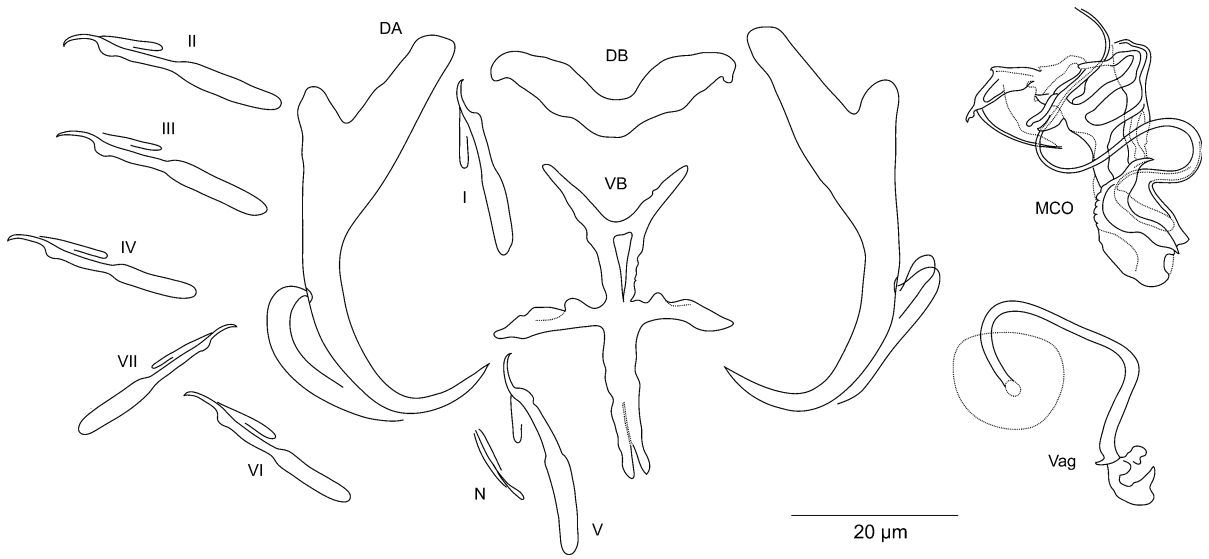

Fig. 6 Sclerotised structures of *Dactylogyrus falsiphallus* n. sp. ex *Luciobarbus maghrebensis*. *Abbreviations*: DA, dorsal anchor; DB, dorsal bar; VB, ventral bar; N, needle; I-VII, hooks (pairs I-V ventral; pairs VI, VII dorsal); MCO, male copulatory organ; Vag, vagina

differentiated from other congeners previously recorded on species of *Luciobarbus* by their anchors having conspicuously enlarged (forma *magnus*) or humped (forma *dromedarius*) inner roots. The presence of humps on the inner roots of anchors in species of *Dactylogyrus* are uncommon, but similar enlargement of the inner roots of anchors has been reported in *D. balistae* Simón Vincente, 1981 from *Barbus bocagei* Steindachner (syn. *Luciobarbus bocagei*) (Simon-Vicente, 1981), *B. comiza* Steindachner (syn. *Luciobarbus comizo*) and *B. sclateri* Günther (syn. *Luciobarbus sclateri*) (El Gharbi et al., 1992) from Spain. However, unlike *D. varius* f. *dromedarius*, *D. ballistae* is a single-bar species of *Dactylogyrus* possessing anchors with inner roots markedly enlarged terminally (*vs* enlarged medially in *D. varius* f. *dromedarius*).

Dactylogyrus varius forma *vulgaris*

[Figs. 5A, 8A.] With characters of species. Measurements, based on 20 specimens, are provided in Table 2. Anchors with inner root slightly expanded basally. *Dactylogyrus varius* f. *vulgaris* is the type-form for the species.

Dactylogyrus varius forma *magnus*

[Fig. 8B.] With characters of species. Measurements, based on 2 specimens, are provided in Table 2. Anchors large, with inner roots evenly expanded along its inner side except terminal portion.

Dactylogyrus varius forma *dromedarius*

[Figs. 5B, 8C.] With characters of species. Measurements, based on 20 specimens, are provided in Table 2. Anchors with inner roots possessing a conspicuous medial hump on their inner side.

Dactylogyrus falsiphallus Rahmouni, Řehulková & Šimková n. sp.

Type-host: *Luciobarbus maghrebensis* Doadrio, Perea & Yahyaoui (Cyprinidae), Maghreb barbel.
Type-locality: River Lahdar (34°14′32.7″N, 4°03′53.9″W), Morocco.
Other localities: River Sebou (34°17′14.2″N, 6°33′14.1″W).
Type-material: Holotype (MNHN HEL575) and 6 paratypes (MNHN HEL575, MNHN HEL576).
Site on host: Gill lamellae.
Representative DNA sequences: GenBank accession numbers: KX553861 (28S rDNA), KX578024 (18S rDNA and ITS1).
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*

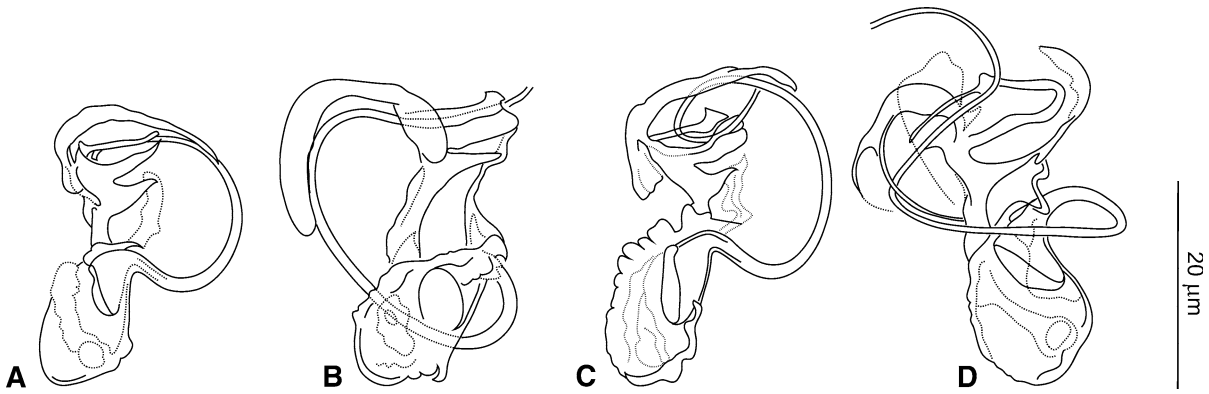

Fig. 7 Drawings of the male copulatory organs taken from specimens analysed molecularly. A, *Dactylogyrus scorpius* n. sp.; B, *Dactylogyrus benhoussai* n. sp.; C, *Dactylogyrus varius* n. sp.; D, *Dactylogyrus falsiphallus* n. sp.

(ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *D. falsiphallus* n. sp. is urn:lsid:zoobank.org:act:6D846081-68FF-4355-B130-461E1BA8809B.

Etymology: The specific epithet (*falsus* = false + *phallos* = penis) reflects the possible confusion of the spike-like distal portion of the accessory piece with the copulatory tube of the MCO.

Description (Fig. 6, 7D)

[Based on 25 specimens.] Body length 500–644 (579); greatest width 110–139 (122) at level of ovary. Haptor with one pair of anchors (dorsal): total length 40–48 (43); length to notch 32–40 (35); inner root 15–19 (16) long; outer root 6–7 (5) long; shaft curved; point extending past level of tip of inner root, 9–11 (9) long. One pair of needles located near hooks pair V. Dorsal bar broadly V-shaped, with narrowed medial part and subterminal notches, 26–34 (31) long. Ventral bar cross-shaped, with five arms, 31–42 (37) long, 22–31 (27) wide. Hooks 7 pairs, similar in shape; each with delicate point, depressed truncate thumb, shank comprised of 2 subunits (proximal subunit expanded); HF loop extending to near level of termination of shank inflation; hook lengths: pair I: 23–26 (24); pair II: 24–26 (25); pair III: 28–33 (30); pair IV: 27–31 (29); pair V: 24–25 (25); pair VI: 24–26 (25); pair VII: 24–27 (25). MCO complex, composed of basally articulated copulatory tube and accessory piece; total length 26–29 (28). Copulatory tube a loose coil following double S-shaped path, distally narrowing to delicate termination, 56–60 (57) long. Accessory piece proximally enclosing base of copulatory tube to form frill-belted capsule-like structure; distal portion recurved, reduced (in its sclerotisation) into long spike following medial part of copulatory tube; medial portion robust, basically with 2 processes: primary process distally articulated to capsule by lightly sclerotised ligament extending to form cap just up to tip of secondary process; secondary process articulated (grooved), with finger-like appendix arising from its base, guiding copulatory tube distally. Vagina a lightly sclerotised wavy tube, with disc-shaped opening, 43–49 (47) long.

Molecular characterisation

The sequence of 28S rDNA was 792 bp long. The sequence of the partial 18S rDNA, entire ITS1 region and partial 5.8S rDNA of *D. falsiphallus* n. sp. was 991 bp long, of which 492 bp corresponded to the 18S rDNA, 487 bp corresponded to the ITS1 region, and 12 bp corresponded to the 5.8S rDNA. Three specimens of *Dactylogyrus falsiphallus* n. sp. from River Lahdar were sequenced, and no intraspecific variability between these specimens was found. Pairwise distances between *D. falsiphallus* n. sp. and three others *Dactylogyrus* spp. analysed using molecular markers are shown in Table 1. Using total pairwise comparisons between the *Dactylogyrus* spp., *D. falsiphallus* was the species with the highest molecular divergence in relation to the three other sepcies described in this study (i.e. molecular divergence of 2.5–3.0% calculated using p-distances).

Remarks

Dactylogyrus falsiphallus n. sp. was collected from the gills of the Maghreb barbel together with specimens belonging to different morphological forms of *D. varius* n. sp. This species shares the same morphological type of MCO with the above described species of *Dactylogyrus*, i.e. *D. scorpius* n. sp., *D. benhoussai* n. sp. and *D. varius* n. sp. However, *D. falsiphallus* n. sp. is differentiated from all these species by its MCO having an accessory piece with a well-developed secondary process terminally covered by a cap of lightly sclerotised ligament (*vs* cap absent in *D. scorpius*, *D. benhoussai* and *D. varius*), a tertiary process modified into a finger-like appendix rising from the base of the secondary process (*vs* a well-developed tertiary process, like a crescent-shaped paddle in the latter species), and a spike-like distal portion (*vs* a foliate/sheath-like distal portion in the latter species).

Discussion

Prior to this study, eight species of *Dactylogyrus* were recorded from ten species of Moroccan *Luciobarbus* (see El Gharbi et al., 1994). The four new species described here raise the number of *Dactylogyrus* spp. to 12 and the number of host species to 12.

In addition to northwestern Africa, species of *Luciobarbus* occur in Iberia, Greece, Anatolia, the Caucasus and the Middle East (Gante, 2011). Thirty-five of the 41 currently recognised species of *Luciobarbus* (see Eschmeyer et al., 2016) are known to be parasitised by species of *Dactylogyrus* (see Gonzalez-Lanza & Alvarez-Pellitero, 1982; El Gharbi et al., 1992; Gussev et al., 1993; Pazooki & Masoumian, 2012; Raissy & Ansari, 2012; Abdullah & Abdullah, 2015). We estimate that 18 species of these monogeneans are characterised by the possession of a cross-shaped ventral bar with five arms. El Gharbi et al. (1994) included Moroccan species of *Dactylogyrus* having a cross-shaped ventral bar and an MCO with a capsule-like base into the 'carpathicus' morphological group. It is worth noting that all of these *Dactylogyrus* species were recorded strictly on *Luciobarbus*. However, three species of *Luciobarbus* (i.e. *L. ksibi*, *L. maghrebensis* and *L. nasus*) were also found to be parasitised by *D. marocanus*, a species belonging to the 'pseudanchoratus' group (see El Gharbi et al., 1994).

Morphologically, all of the species of *Dactylogyrus* described in this study are categorised as members of the 'carpathicus' group. Furthermore, despite the impossibility of re-examination of type-specimens, the basic structure of the MCO of at least four of the previously described *Dactylogyrus* spp., i.e. *D. atlasensis*, *D. borjensis*, *D. ksibii* and *D. ksibioides*, suggests a close relationship to the species of *Dactylogyrus* newly described in the present paper. The MCO in all of these species is characterised by an accessory piece with a distal part bending inward as a guide for the copulatory tube. In view of the scorpion-like curved tail appearance of the accessory piece, we refer to the above previously-described and four new species of *Dactylogyrus* as members of the 'scorpion' subgroup. The MCOs of *D. scorpius* n. sp., *D. benhoussai* n. sp., *D. varius* n. sp. and *D. falsiphallus* n. sp. exhibit the same morphological pattern, including an accessory piece characterised by a complex medial part from which three processes arise. The MCOs of *D. benhoussai* n. sp. and *D. varius* n. sp. are in fact so similar (cryptic) that they are generally insufficient for separating the two species on morphological grounds. Also, except for *D. varius* f. *magnus* and *D. varius* f. *dromedarius*, all the presently described new species of *Dactylogyrus* exhibit small morphological differences in their haptoral structures.

However, as indicated above, three morphological forms (based on the morphology of the haptoral anchors) were recognised within *D. varius* n. sp. Interestingly, all of these forms can co-occur on the same host specimen, but we have no data concerning their spatial distribution patterns on the different gill arches. Previous studies documented some variability in the haptoral sclerites of monogeneans only in relation to biogeographical distribution (Rohde & Watson, 1985; Boeger & Kritsky, 1988; Vignon & Sasal, 2010). However, as far as we are aware, our study provides the first evidence that specimens of one monogenean species parasitising a single host specimen show significant shape variability in the haptoral anchors. As the three morphological forms do not exist under the traditional concept of a subspecies, i.e. being a geographically circumscribed population (Mayr &

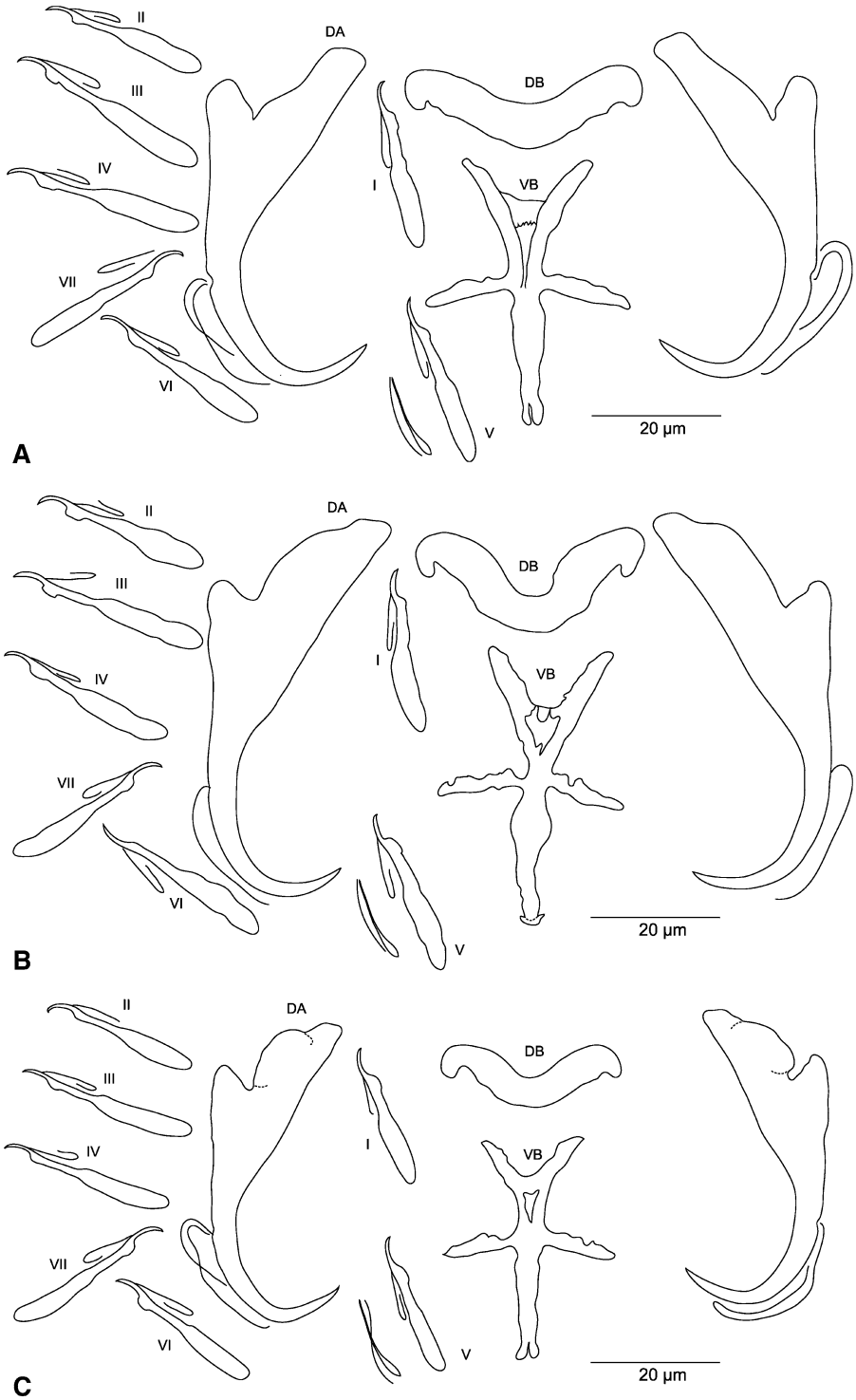

Fig. 8 Drawings of haptoral structures taken from specimens of *Dactylogyrus varius* n. sp. analysed molecularly. A, forma *vulgaris*; B, forma *magnus*; C, forma *dromedarius*

Table 2 Measurements of three morphological forms of *Dactylogyrus varius* n. sp. from *L. maghrebensis*

Dactylogyrus varius	f. *vulgaris* (n = 20)	f. *magnus* (n = 2)	f. *dromedarius* (n = 20)
Body length	322–600 (495)	778, 780	500–795 (702)
Body width	66–100 (99)	195, 200	100–170 (127)
Anchors			
1	41–56 (48)	57, 61	40–55 (48)
2	32–42 (37)	46, 50	32–42 (37)
3	17–22 (18)	21, 25	14–20 (18)
4	3–7 (6)	6, 8	5–7 (6)
5	8–10 (9)	9, 11	9–11 (10)
Ventral bar			
lenght 6	31–39 (35)	41, 45	30–43 (36)
width 7	25–30 (28)	28, 30	23–31 (26)
Dorsal bar			
length 8	30–35 (31)	32, 36	27–35 (31)
Hooks length 9			
I	23–27 (25)	25, 29	20–26 (24)
II	20–25 (24)	30, 32	24–29 (27)
III	26–35 (29)	31, 33	28–34 (30)
IV	25–31 (29)	30, 34	28–33 (31)
V	22–26 (25)	27, 29	22–27 (26)
VI	21–29 (27)	29, 31	24–29 (28)
VII	26–30 (28)	28, 30	26–32 (29)
Vagina length 10	40–51 (46)	50, 54	40–48 (44)
MCO length 11	24–30 (27)	29, 33	26–31 (28)

Ashlock, 1991; Futuyma, 1998), we termed these morphotypes as forms in this study.

The sequences of partial 18S ribosomal DNA, the entire first internal transcribed spacer (ITS1), and 28S ribosomal DNA were used to estimate the level of molecular divergence between the new species described in this study. Our molecular analyses support the distinct species status for all four newly described *Dactylogyrus* spp. from Moroccan *Luciobarbus* spp. and highlight the need to use molecular analyses when investigating almost morphologically indistinguishable congeneric parasites infecting hosts evolving by rapid speciation. In addition, our study demonstrates the usefulness of molecular markers when facing high phenotypic polymorphism concerning the sclerotised parts of the haptor, as shown in the case of *Dactylogyrus varius* n. sp. Three forms of *Dactylogyrus varius* n. sp. differing in the morphology of their haptoral anchors exhibited no nucleotide variability using any of the molecular markers applied in this study, which seems to support the view that this variability may potentially represent some ontogenetic changes in the haptoral sclerites.

Šimková et al. (2004) calculated pairwise uncorrected p-distances for 51 *Dactylogyrus* species parasitising European cyprinid species using combined sequence data from partial 18S rDNA and the ITS1 region. They showed that the most phylogenetically closely-related *Dactylogyrus* species parasitising the same cyprinid species exhibited 1.4% of molecular divergence (this value can be considered as genetic threshold for morphologically differentiated *Dactylogyrus* spp.), while the molecular divergence between other pairs of *Dactylogyrus* spp. was more than 2.6%. In our study, the lowest level of molecular divergence calculated for combined sequence data from 18S rDNA and ITS1 was 1.5% between two morphologically similar (cryptic) species, i.e. *D. varius* n. sp. and *D. benhoussai* n. sp. For other species pairs, the molecular divergence ranged from 2.5 to 3.8%. The highest level of genetic divergence between the pairs of *Dactylogyrus* spp. described in our study was recorded using ITS1 sequences (i.e. 2.9–7.2%) representing fast evolving regions useful for the phylogenetic reconstruction of closely related *Dactylogyrus* with rapid speciation, i.e. especially *Dactylogyrus* infecting cyprinid species of the same phylogenetic lineage (Šimková et al., 2004). Overall, the morphologically similar (cryptic) species *D. scorpius* n. sp., *D. benhoussai* n. sp. and *D. varius* n. sp. showed smaller molecular divergence using all the analysed markers in our study. In contrast, morphologically well-differentiated *D. falsiphallus* n. sp. exhibited the highest level of molecular divergence when compared to the other three *Dactylogyrus* spp., evidenced especially for the ITS1 region. Concerning the partial sequences of 18S ribosomal DNA analysed in our study, the level of molecular divergence was relatively weak (ranging from 0.2 to 0.6%). This marker represents a conserved gene evolving relatively slowly (Hillis & Dixon, 1991). Previous phylogenetic studies of *Dactylogyrus* parasites showed the suitability of both 18S and 28S rRNA genes for the resolution of the phylogenetic relationships between species of *Dactylogyrus* from different phylogenetic lineages of cyprinids (Šimková et al., 2004, 2006). The pairwise genetic distances calculated between species described in this study

using partial sequences of 28S rDNA ranged from 0.4 to 2.4% and also support a higher degree of molecular divergence (≥ 2%) between *D. falsiphallus* n. sp. and the three *Dactylogyrus* spp. exhibiting more similar morphologies of their MCOs than between pairs of these morphologically similar species as revealed using analyses of the ITS1 region.

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Compliance with ethical standards

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

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed.

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