An integrative taxonomic approach to reveal the status of the genus *Pomphorhynchus* Monticelli, 1905 (Acanthocephala: Pomphorhynchidae) in Austria

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ABSTRACT

Species of the genus *Pomphorhynchus* Monticelli, 1905 (Acanthocephala: Pomphorhynchidae) are obligate endoparasites infesting mostly freshwater fish. Morphological identification is challenging due to high intraspecific variations. The use of molecular analyses enabled new insights into the diversity and revealed high cryptic presence and unknown distribution patterns for various European species. In Austria only one species, *Pomphorhynchus laevis* (Müller, 1776), has been reported so far. We conduct an integrative analysis of *Pomphorhynchus* in Austria with a combination of morphological and molecular methods. Our results revealed the presence of three species of *Pomphorhynchus* in Austrian waters: *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis* (Rudolphi, 1809) and *Pomphorhynchus bosniacus* Kiskároly and Čanković, 1967. While *P. bosniacus* was the predominant species in the Danube, *P. laevis* was recorded exclusively in Styria. *Pomphorhynchus tereticollis* occurred mainly in rivers of Styria except for one individual found in the Danube. We document the first occurrence of *P. bosniacus* and *P. tereticollis* in Austria. We found a high intraspecific haplotype variation in *P. bosniacus* suggesting that the species has a longer history in Central and Western Europe. It was previously misidentified as *P. laevis*, which is also true for *P. tereticollis*. A large number of hosts examined were infected with only juvenile and cystacanth stages suggesting paratenic infections. Our study highlights the importance of using an integrative taxonomic approach in the identification of species of *Pomphorhynchus*.

1. Introduction

The acanthocephalans in the genus *Pomphorhynchus* Monticelli, 1905 are obligate parasites of freshwater fish and, less frequently, of marine fish, amphibians and mammals (Meyer, 1933; Kennedy, 1984; Dimitrova et al., 2008). Previous studies dealt with their ecology, morphology and geographical distribution throughout Europe, with particular focus on *Pomphorhynchus laevis* (Müller, 1776) and *Pomphorhynchus tereticollis* (Rudolphi, 1809). Besides *P. laevis* and *P. tereticollis* parasitizing teleosts in Europe, *Pomphorhynchus kostylevi* Petrochenko, 1956 from *Capoeta sevangi* (de Filippi, 1865) (Cyprinidae) (Gibson et al., 2014), and *Pomphorhynchus bosniacus* Kiskároly and Čanković, 1967 from various fish species were reported from rivers and lakes in the Balkans (Kakacheva-Avramova, 1973; Hristovski, 1999;

Cakic et al., 2008). A fifth European species, *Pomphorhynchus intermedius* Engelbrecht, 1957 is controversially discussed. Initially it was treated as *P. laevis f. intermedius* in the Greifswalder Bodden, Germany (Engelbrecht, 1957). Later it was elevated to species level by Golvan (1969) and in 2003 again classified as a synonym of *P. laevis* by Amin et al. (2003). It is listed as valid species in the latest classification of Acanthocephala (Amin, 2013), although Špakulová et al. (2011) regarded *P. intermedius* as a synonym of *P. tereticollis* due to similarities of the extensions on the hook roots. However, also the taxonomic status of *P. tereticollis* was repeatedly discussed – first described by Rudolphi (1908) as *Echinorhynchus tereticollis*, it was classified as synonym of *P. laevis* for a long time (Engelbrecht, 1957; Amin et al., 2003). However, DNA sequence analyses supported the species status of *P. tereticollis* (Perrot-Minnot, 2004; Bombarová et al., 2007) and finally it was re-

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described based on morphological characters and molecular genetic data (Špakulová et al., 2011).

As Pomphorhynchidae in general exhibit only few differentiating morphological characters and show a high intraspecific variability, species identification based on morphological characters alone remains challenging (Kennedy, 2006; Špakulová et al., 2011; Hohenadler et al., 2017; Li et al., 2017). Due to the morphological similarity between *P. laevis* and *P. tereticollis* they might have been repeatedly misidentified, which possibly led to an underestimation of the distribution and abundance of *P. tereticollis* throughout Europe (Emde et al., 2012; Hohenadler et al., 2017). In recent years, the use of molecular genetic methods in the characterization of *Pomphorhynchus* spp. allowed new insights into the diversity, phylogenetic relationships and geographical occurrences of various species. For example, Perrot-Minnot et al. (2018) found two genetic strains of *P. tereticollis*, one mostly infecting marine fish and the other one infecting mostly cyprinid fish. Various genetic lineages of *P. laevis* were detected that vary in microhabitat (within the intestine) and show slight morphological differences (Dudiňák and Šnábel, 2001; O'Mahony et al., 2004; Vardić Smrzlić et al., 2015). Recently, Perrot-Minnot et al. (2018) differentiated five genetic lineages of *P. laevis* in Europe (from central and eastern parts of the Northern Mediterranean, Western and Central Europe to Ponto-Caspian (P.-C.)) with genetic distances (Kimura-2-parameter) from 10.5% to 20.3%. The genetic differentiation of these lineages was related to their geographic distribution.

However, less is known about the status of the genus *Pomphorhynchus* in Austria. The latest survey of Austrian Acanthocephala dates back more than 30 years ago when Kritscher (1985) listed *P. laevis* as the only representative of the genus in Austrian waters. Laimgruber et al. (2005) identified *P. laevis* as the predominant helminth infesting the common barbell *Barbus barbus* (Linnaeus, 1758) in the Austrian parts of the rivers Danube and Drau. These surveys in Austria were based on morphological methods. However, recently five sequences of *P. laevis* were published obtained from *B. barbus* and the round goby *Neogobius melanostomus* (Pallas, 1814) collected in the Austrian part of the Danube (Perrot-Minnot et al., 2018). These sequences belong to a lineage of *P. laevis* described from P.-C. Europe by Perrot-Minnot et al. (2018) and used also by David et al. (2017). However, no information was provided regarding the morphology of these specimens and the determination was based on DNA data solely compared to those processed by Špakulová et al. (2011).

Considering the high diversity of genetic lineages detected so far as well as the morphological similarities, we performed a survey in fish derived from Austrian waters of Vienna, Lower Austria, Upper Austria, Carinthia and Styria for the presence of species of *Pomphorhynchus*. Our aim was to conduct a thorough morphological investigation of specimens using light microscopy, confocal laser scanning microscopy and 3-D reconstruction of histological sections. The morphological investigation with light microscopy is limited, as the main distinguishing morphological characters of *P. tereticollis* and *P. laevis*, namely the extensions on the basal hooks on the proboscis, are visible in living specimens, but become unrecognizable by conventional whole mount analyses after fixation (Špakulová et al., 2011; Hohenadler et al., 2017). Therefore, we established a novel approach for the morphological identification of *Pomphorhynchus* species by means of 3-D reconstructions of histological serial sections. Second, the specimens were analyzed by molecular genetic methods to determine a partial section of the *cytochrome c oxidase subunit I* (*COI*), the so-called DNA barcode region. So far, this is the first study in Austria to use such an integrative taxonomic approach to identify species of *Pomphorhynchus*.

2. Material and methods

2.1. Host and parasite sampling

A total of 162 fish specimens from different rivers and lakes of Styria, Vienna, Carinthia, Upper Austria and Lower Austria were examined (Table 1). Fresh material was either preserved in 80% ethanol, frozen or dissected immediately after capture. Most of the fish intestines were derived from fish investigated during the *Austrian Barcode of Life* (ABOL) pilot study "DNA barcoding of Austrian vertebrates". The parasites were obtained by dissection of fish intestines. Parasites were preserved in 80% ethanol and temporarily stored at 4 °C for further investigations. Specimens could be macroscopically assigned to the genus *Pomphorhynchus* based on the long neck and bulb. A total of 39 individuals of *Pomphorhynchus* ssp. were analyzed morphologically as well as genetically. In addition, nine specimens were analyzed only morphologically and 16 only genetically (Table 1).

Generated DNA barcodes were uploaded to the Barcode of Life Database (BOLD) (Accession numbers: **ACANT001-17**, as well as **ACANT002-18 – ACANT049-18**).

2.2. Morphological identification

2.2.1. Staining and clearing techniques

For morphological identification of preserved specimens with a light microscope and a confocal laser scanning microscope, clearing in glycerol is necessary (Lühe, 1911). For clearing, specimens were transferred into a solution of 80% ethanol and glycerol (proportion 50:50) in an embryo dish and incubated for at least one day at 38 °C until the ethanol evaporated (Reichenow et al., 1969). Subsequently, the samples were mounted in glycerol for microscopic analysis.

2.2.2. Sectioning and 3-D reconstruction

To reconstruct important morphological characters such as the extensions of the basal hooks, 3-D reconstructions of histological semithin sections were performed. Therefore, proboses of the different species were cut from the trunk and rapidly dehydrated via acidified dimethoxypropane (DMP). Then they were embedded into Agar LVR resin (Agar Scientific, Stansted, Essex, UK) via acetone as intermediate. Serial semithin sections of cured resin blocks were prepared with a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) at a thickness of 1 μm. Sections were stained with toluidine blue and subsequently examined on a Nikon NiU light microscope (Nikon Instruments, Tokyo, Japan) equipped with a Ds-Ri2 microscope camera. Image stacks from the serial sections were converted to greyscales and imported into the 3D reconstruction software Amira 6.11 (FEI, Hillsboro, Oregon, USA). The general outline of the proboscis was displayed by using a volume rendering of the grey-scale information contained in the histological series (Handschuh et al., 2010). Segmentation of single hooks was conducted manually with the brush tool. A surface of the segmented hooks was generated (Ruthensteiner, 2008) and combined with the volume rendering for a general outline. Snapshots were taken with the Amira 6.11 software (FEI, Hillsboro, Oregon, USA). At least one longitudinal row of hooks was reconstructed for each of the five specimens.

2.2.3. Light microscopy

Microphotographs were taken with a Nikon Eclipse E600 light microscope (Nikon, Chiyoda, Tokio, Japan) equipped with an Olympus DP27 microscope camera (Shinjuku, Tokio, Japan). Subsequent image processing was done in Adobe Photoshop CC (Adobe Systems Software Ireland Ltd.) and image stacks were produced with the software Zerene Stacker (Zerene Systems LLC, Richland).

2.2.4. Confocal laser scanning microscopy

Analysis of autofluorescence spectra of different excitation wavelengths was conducted with a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Depending on the objective different z-step sizes from 0.5 μm to 1 μm were used. Image stacks were merged into maximum intensity projection images.

The confocal microscope image stacks were imported and processed

Table 1
List of all examined specimens of this study (all from Austria).

Lab-ID	BOLD-Process-ID	method	Host family	Host species	Geographic origin
P. bosniacus					
DK1-1	**ACANT002-18**	LM, M, G	Cyprinidae	*Leuciscus idus*	Danube, Vienna
DK1-3	**ACANT003-18**	LM, M, S, G	Cyprinidae	*Leuciscus idus*	Danube, Vienna
DK1-4		LM, M	Cyprinidae	*Leuciscus idus*	Danube, Vienna
FA7-1		LM, M	Cyprinidae	*Leuciscus idus*	Danube, Vienna
FA8-1	**ACANT004-18**	LM, G	Cyprinidae	*Squalius cephalus*	Danube, Vienna
FA9-1		LM	Cyprinidae	*Squalius cephalus*	Danube, Vienna
FA9-3	**ACANT005-18**	LM, M, CLSM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-4	**ACANT006-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-6	**ACANT007-18**	LM, M, G, S	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA01G-1	**ACANT008-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA02G-1	**ACANT009-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA03G-1	**ACANT010-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA04K-1	**ACANT011-18**	G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA05K-1	**ACANT012-18**	G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA06K-1	**ACANT013-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA07F-1	**ACANT014-18**	G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA09F-1	**ACANT015-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-DA12FK-1	**ACANT016-18**	G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA01G-1	**ACANT017-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA02G-1	**ACANT018-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA03G-1	**ACANT019-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA04K-1	**ACANT020-18**	G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA07F-1	**ACANT021-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA08F-1	**ACANT022-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-MA09F-1	**ACANT023-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-NA01OH-1	**ACANT024-18**	LM, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S1		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S2		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S3		LM, M	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S5		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S6		LM, M	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-N7	**ACANT046-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S8		LM, M	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S10		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-S11		LM, M	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-T2	**ACANT049-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-T4		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-T7	**ACANT047-18**	LM, M, G, S	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-T8		LM, M	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-N6	**ACANT048-18**	LM, M, G	Cyprinidae	*Barbus barbus*	Danube, Vienna
FA9-N12		LM, M, G*	Cyprinidae	*Barbus barbus*	Danube, Vienna
P. tereticollis					
Fish26-1	**ACANT025-18**	G	Percidae	*Zingel streber*	Mur, Styria
Fish29-2	**ACANT001-17**	CLSM, S, G	Cyprinidae	*Alburnus alburnus*	Mur, Styria
Fish32-2	**ACANT026-18**	G	Salmonidae	*Thymallus thymallus*	Mur, Styria
Fish36-2	**ACANT027-18**	LM, G	Cyprinidae	*Alburnoides bipunctatus*	Mur, Styria
Fish39-1	**ACANT028-18**	G	Cyprinidae	*Pseudorasbora parva*	Mur, Styria
Fish42-1	**ACANT029-18**	CLSM, G	Cyprinidae	*Barbus barbus*	Mur, Styria
Fish45-5	**ACANT030-18**	G	Lotidae	*Lota lota*	Mur, Styria
Fish45-8	**ACANT031-18**	LM, G	Lotidae	*Lota lota*	Mur, Styria
Fish45-9	**ACANT032-18**	LM, G	Lotidae	*Lota lota*	Mur, Styria
Fish45-10[+]	**ACANT033-18**	G	Lotidae	*Lota lota*	Mur, Styria
Fish45-11[+]	**ACANT034-18**	G	Lotidae	*Lota lota*	Mur, Styria
Fish45-12		LM	Lotidae	*Lota lota*	Mur, Styria
Fish308-1	**ACANT035-18**	LM, G	Percidae	*Zingel streber*	Mur, Styria
FA1-1	**ACANT036-18**	LM, G	Cyprinidae	*Alburnus alburnus*	Danube, Vienna
P. laevis					
Fish34-1[+]	**ACANT037-18**	G	Cyprinidae	*Alburnoides bipunctatus*	Mur, Styria
Fish34-2[+]	**ACANT038-18**	G	Cyprinidae	*Alburnoides bipunctatus*	Mur, Styria
G1-1	**ACANT039-18**	LM, G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G2-2	**ACANT040-18**	LM, G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G3-1	**ACANT041-18**	LM, G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G3-2[+]	**ACANT042-18**	G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G3-3[+]	**ACANT043-18**	G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G3-5[+]	**ACANT044-18**	G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria
G4-1	**ACANT045-18**	LM, S, G	Cyprinidae	*Alburnoides bipunctatus*	Sulm, Styria

*sequences not included in this study.

[+]cystacanths.

Abbreviations: LM = light microscopy, M = documented measurements, S = sectioned (and 3-d reconstructed), CLSM = confocal laser scanning microscopy, G = molecular genetics.

Fig. 1. Map showing European rivers relevant for the present study. The study area in Austria is highlighted in grey. Localities of *P. bosniacus* are indicated as circle, the one of *P. tereticollis* as asterisk and the one of *P. laevis* as square. Different sizes are indicating higher or lower abundances.

in *FIJI* (Schindelin et al., 2012). Subsequent volume rendering was done with the reconstruction and visualization software Amira 6.11 (FEI, Hillsboro, Oregon, USA).

2.2.5. Measurements

Classification was based on species-specific morphological characters according to literature (Lühe, 1911; Meyer, 1933; Petrochenko, 1956; Golvan, 1969; Špakulová et al., 2011). Trunk size, neck length, bulb diameter, proboscis length, hook length, testes, cement glands and eggs were measured. Measurements were performed in NIS Elements (Nikon Instruments Europe BV, Amsterdam, Netherlands) and AMIRA 6.11 and compared with the data of the classification literature.

2.3. Molecular genetic analysis

2.3.1. DNA extraction, amplification and sequencing

DNA extraction was conducted in a clean room using the QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the protocol of the manufacturer. For DNA barcoding a partial sequence (amplicon size 711 bp) of the mitochondrial *cytochrome c oxidase unit 1* gene (*COI*) was used. The following primer pairs were used for amplification: H14AcanCOIFw1 (5- TTCTACAAATCATAARGATATYGG) as forward primer and H14AcanCOIRv2 (5- AAAATATAMACTTCAGGAT GACCAAA) as reverse primer. The nested forward primer Pompho-1 + (AGACTACTAATTCGATTAGA) was designed for the species *P. laevis* due to problems in the sequence reactions. PCR reactions were performed in a final volume of 25 μl containing 18.9 μl distilled water, 2.5 μl 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.5 units TopTaq Polymerase and 1 μl template DNA. Amplification started with an initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 48 °C for 60 s and extension at 72 °C for 60 s. Finally, to complete elongation, the temperature was held at 72 °C for 7 min. The PCR products were sequenced (both directions) by Microsynth (Balgach, Switzerland) using the PCR primers.

2.3.2. Alignments and calculations of sequence distances

Sequences were edited and aligned using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall, 1999). No insertions or deletions were observed, and the reading frame of the sequences proved to be intact. Thus, there was no indication for unintended amplification of NUMTs (nuclear pseudogenes of mt sequences).

For comparison the following NCBI GenBank sequences were included into the alignment:

AY218096, AY423348–AY423353, EF051062–EF051071, KF559284–KF559300, LN994875–LN995000, LN994844–LN994873, LN994840–LN994842, JF706706, JN695504– JN695508 and **MF563497–MF563527**. A published sequence of *Echinorhynchus truttae* Schrank, 1788 (**DQ089710**) was used as outgroup to root the tree. Due to the fact that the sequences from GenBank had a shorter length we trimmed the sequences produced in the present analyses to the same length (550 bp). MEGA v7 (Kumar et al., 2016) was used to calculate Neighbour Joining trees (*NJ*), performing 1000 bootstrap replicates in order to assess branch support. Further graphical processing of the tree was done in FigTree 1.4.3 (http://tree.bio.ed.ac.uk) and InkScape 0.92 (https://inkscape.org). Inter- and intraspecific *p*-distances were calculated with MEGA v7 (Kumar et al., 2016).

Median Joining haplotype networks (Bandelt et al., 1999) were produced with the software PopART 1.7 (http://www.popart.otago.ac.nz). Networks were graphically processed in InkScape 0.92 (https://inkscape.org). The haplotypes were classified according to their collection countries. Calculations of haplotype diversity (Hd) and nucleotide diversity (π) of the *COI* dataset were conducted in DnaSP v5 (http://www.ub.edu/dnasp; Librado and Rozas, 2009).

3. Results

Overall, nine different fish species belonging to four families were infested with parasites of the genus *Pomphorhynchus*. In total 239 specimens of the genus *Pomphorhynchus* were collected from 18 infested fish specimens. Five of these fish were collected in the Viennese part of the Danube and 14 fish were collected in the rivers Mur and Sulm in Styria (Table 1). Localities are visualized in Fig. 1.

3.1. Morphological analysis

Three different species of *Pomphorhynchus* were morphologically distinguished in this study.

3.1.1. Pomphorhynchus laevis

Juvenile specimens obtained from *Alburnoides bipunctatus* (Bloch, 1782) of the river Sulm (Styria) were identified as *P. laevis*. Four specimens were examined under the light microscope since the hooks exhibited no autofluorescence in the confocal scans. They had a
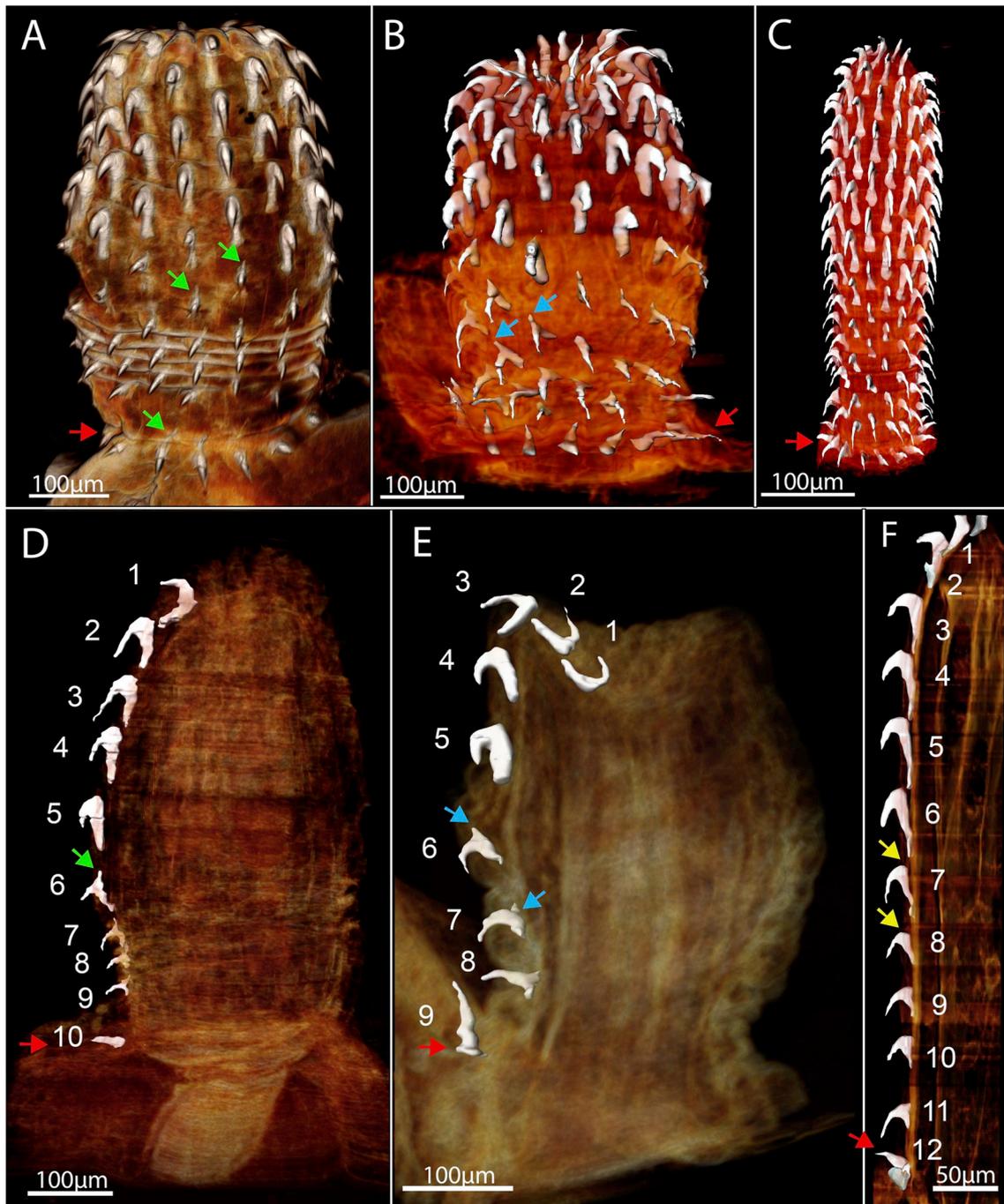
Fig. 2. Proboscis and longitudinal row of hook roots of the three determined *Pomphorhynchus* species. All images are volume rendering of the 3-D stacks. In volume rendering each grayscale value is assigned a color according to an arbitrary colormap. The depiction depends on which values and how transparent they are displayed. In the present images a glow color map is used, which assigns graded colors from black (background) to shades of red-orange-yellow. **A.** Volume rendering of a confocal laser scan image stack of the proboscis of *P. tereticollis*. **B.** Volume rendering of the sectioned proboscis of *P. bosniacus* including reconstructed hooks as surface rendering. **C.** Volume rendering of the sectioned proboscis of *P. laevis* including reconstructed hooks as surface rendering. **D-F.** Internal view of the sectioned proboscids including a single longitudinal row of hooks. **D.** *P. tereticollis*. **E.** *P. bosniacus*. **F.** *P. laevis*. Legend: Red arrow = last circle of hooks; green arrow = extensions on basal hook roots of *P. tereticollis*; blue arrow = basal hooks attached in the middle of hook roots of *P. bosniacus*; yellow arrow: lack of extensions on basal hook roots of *P. laevis*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cylindrical proboscis with twelve hooks per row. The 3-D reconstruction of one specimen emphasized the assignment to the species *P. laevis* since no extensions on the basal hooks of the proboscis were visible as it was described by Meyer (1933) and Špakulová et al. (2011) (Fig. 2 C, F). The anterior and basal hooks exhibit long hook roots (Fig. 2 C). The furcation of the basal roots described by Meyer (1933) could not be confirmed in the 3-D reconstruction since the thicker outer parts are interconnected by a very thin matrix. Also, the basal circle of hooks is located on the posterior part of the proboscis as it is known for *P. laevis* (Meyer, 1933; Špakulová et al., 2011; Hohenadler et al., 2017). Overall, 17 longitudinal rows of hooks were counted.

3.1.2. Pomphorhynchus tereticollis

Eight juvenile specimens obtained from different fish species in Styria (Table 1) were assigned to the species *P. tereticollis*. Since Špakulová et al. (2011) provided data of juvenile specimens of the

Table 2
Ranges of measurements conducted on 17 female (five adult, twelve subadult) and nine male (five adult, four subadult) specimens of *P. bosniacus*. Since adults and subadults differed only in trunk size, the remaining measurements were combined for both life stages. Averaged measurements are given in parentheses. Measurements are in μm unless otherwise stated.

P. bosniacus	female		male	
	subadult	adult	subadult	adult
trunk (mm)	6.5 – 9.5 (8.4) × 1.2 – 1.6 (1.3)	10.1 – 18.2 (12.09) × 1.2 – 1.6 (1.4)	4.2 – 6.2 (5.7) × 1.1 – 1.5 (1.3)	8.1 – 10.9 (9.1) × 1.2 – 1.5 (1.3)
neck (mm)	2.1 – 3.1 (2.21)		0.58 – 2.58 (1.7)	
bulb	475 – 792 (653) × 316 – 1,132 (529)		363 – 842 (654) × 143 – 998 (435)	
proboscis	563.94 – 724.84 (656.53) × 210 – 260 (240)		616.7 – 698.21 (651.9) × 220 – 280 (250)	
lemnisci length	839.97 – 1265.34 (1205.68) × 165.2 – 192.3 (176.43)		239.05 – 957.19 (682.36) × 109.2 – 178.5 (162.28)	
testes (n = 5)			283.65 – 1243-56 (799.8) × 156.2 – 457.79 (352)	
cement glands (n = 5)			426.72 – 619.06 (508.62)	
hook length				
hook #1	43.28 – 58.65 (54.11)		46.96 – 58.67 (54.11)	
hook #2	50.25 – 64.46 (62.08)		60.58 – 68.57 (61.73)	
hook #3	53.52 – 67.49 (61.78)		61.3 – 67.84 (61.78)	
hook #4	47.14 – 58.91 (53.69)		49.44 – 59.17 (52.69)	
hook #5	43.58 – 57.3 (51.24)		47.45 – 58.86 (50.24)	
hook #6	31.53 – 46.99 (39.14)		35.25 – 45.21 (39.14)	
hook #7	33.41 – 45.21 (38.3)		33.32 – 47.99 (38.3)	
hook #8	32.77 – 44.9 (39.18)		30.09 – 51.89 (38.84)	
hook #9*	31.71 – 41.22 (36.29)		29.15 – 41.89 (36.29)	

*not present in every specimen.

original Rudolphi type material in their re-description of *P. tereticollis,* we were able to compare our results even though we only had juvenile material at hand. The assignment was not possible with conventional light microscopy as the extensions on the basal hooks of the proboscis were not visible after fixation. Subsequently, confocal laser scans were performed, which visualized the species-specific extensions on the basal hooks and showed the posterior branching of the basal hook roots (Fig. 2 A, Fig. S1). Remarkably, this species was the only representative of the genus whose hooks were autofluorescent. Therefore, for a better comparability with the other two species, we performed a 3-D re-construction of a hook row of the sectioned proboscis (Fig. 2 D). This method also showed the extensions on the basal hooks with exception of basal hook no. 10 for which it was not possible to depict the extension in the general view (Fig. 2 D). However, the extensions of these last hooks are visible in Fig. 2 A and Fig. S1. The examined specimen had 16 longitudinal rows of hooks on the proboscis. The proboscis had an ovoid shape armed with ten hooks per row. The five anterior hooks were stout while the four basal hooks were smaller. Last circle of basal hooks was located on the anterior part of the bulb, a species-specific trait known for *P. tereticollis* (Špakulová et al., 2011).

3.1.3. Pomphorhynchus bosniacus

Adult and subadult specimens of a morphologically different species were found in fish obtained from the Danube (Table 1). This species was found in high numbers (n = 186) in *Barbus barbus* and in one individual a mass occurrence ("polyhelminthiasis") was observed. Moreover, subadult specimens belonging to this species were obtained from other fish species (Table 1). Twenty-six specimens were examined and measured. The proboscis had a cylindrical shape (Fig. 2 B). The five anterior hooks were stouter than the posterior ones and their number was constant in each individual, while the number of the posterior hooks varied from three to four hooks per row (Fig. 2 E). The basal hooks on the proboscis were attached in the center of the root. The hook roots were also visible under the light microscope in fixed material, while, e.g., the hook root extensions of *P. tereticollis* were not visible after fixation. Hooks number four and five had a clearly stouter appearance than the anterior three hooks (Fig. 2 B). The last circle of hooks was located on the anterior part of the bulb (Fig. 2 B). The number of longitudinal rows of hooks differed from 14 to 16 longitudinal rows (in

three sectioned specimens). The females were slightly larger than the males. Table 2 provides measurements of trunk size, neck length, bulb diameter, proboscis length and hook length from 17 female specimens (five adults, twelve subadults) and nine males (five adults, four sub-adults). Furthermore, in several specimens additional measurements of lemnisci, testes and cement glands were taken (Table 2). Eggs were elliptic in shape and were 83.4–89.1 (86.5) μm × 15.5–18.9 (16.5) μm. Furthermore, three different neck bulb shapes were determined as described by Kiskároly and Čanković (1967). Although the specimens of this study were slightly smaller in size than *P. bosniacus* described by Kiskároly and Čanković (1967), the size and arrangement of the hooks, the length of the proboscis and the bulb variations (Kiskároly and Čanković, 1967; Moravec, 2004) suggest that the Austrian specimens of the Danube belong to the same species.

3.2. Molecular genetic results

3.2.1. Neighbour-joining tree

A 550 bp sequence of the mitochondrial *COI* was analyzed in 49 specimens. The *NJ* tree calculated from the *COI* dataset including also 203 sequences from GenBank obtained high support values for most of the nodes (Fig. 3 A). Six major clades were found, two of which were divided into subclades (Fig. 3 A). The same clades were found already by David et al. (2017) and part of them by Perrot-Minnot et al. (2018), although the relationships among clades were slightly different in those three trees.

The basal split in the tree separates *P. tereticollis* from the rest. This clade contains sequences from the present study, morphologically identified as *P. tereticollis*, as well as sequences determined as *P. tereticollis* obtained from NCBI GenBank. From the next three nodes in the tree two Italian clades (one from the Adriatic, the other one from the Tyrrhenian Sea) and a clade with samples from Turkey branch off, they all contain exclusively sequences from GenBank. The remaining main clades (designated here clade I and II) are sister groups, each sub-divided into subclades and each containing samples analyzed in the present study.

In the first main clade (I) one subclade contains specimens morphologically identified in the present study as *P. bosniacus* as well as published GenBank sequences from specimens of P.-C. Europe/Danube
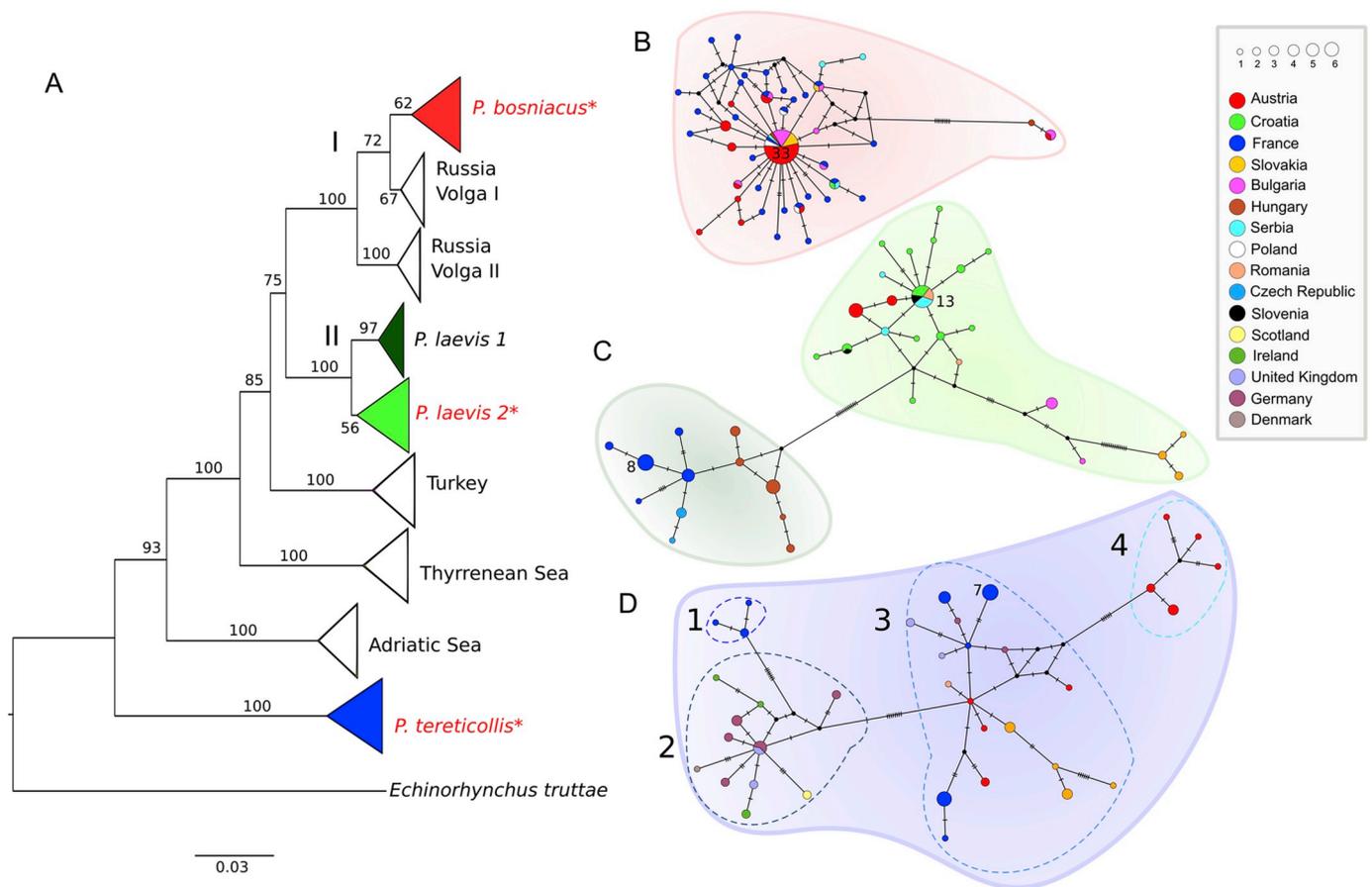
Fig. 3. Phylogenetic relationships between species of the genus *Pomphorhynchus* based on a 550 bp *COI* dataset. **A.** *NJ* tree showing uncorrected *p*-distances between species of the genus *Pomphorhynchus*. Bootstrap values (1000 replicates, in %) are shown next to the nodes. Clade names containing specimens examined in this study are colored in red and marked with an asterisk. The dataset includes sequences generated in this study and sequences obtained from NCBI GenBank. **B.** Median-joining network of *P. bosniacus*. One frequent haplotype can be observed (n = 33). Most haplotypes are constituted of 1 sample (see legend), separated by one to two mutation steps from the main haplotype. Only two haplotypes including sequences from NCBI GenBank are separated by eight mutation steps from the main haplogroup. **C.** Median-joining network of *P. laevis*. Two different clades can be distinguished: a Western clade (dark green) and an Eastern clade (light green). Specimens of this study are represented in two separate haplotypes. **D.** Median-joining network of *P. tereticollis*. Four different haplogroups can be distinguished, indicated with dotted lines: **1.** Specimens from France (Rhône) (NCBI GenBank), **2.** Specimens from Northern Europe (NCBI GenBank), **3.** Specimens from the Rhine and Carpathians (NCBI GenBank), including specimens of this study, **4.** Specimens of this study. Mutation steps are indicated with vertical lines. Black dots represent haplotypes missing in the study sampling. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and from the Upper Rhine in France, determined as *P. laevis* (Fig. 3 A). This subclade is closely related to two subclades comprising specimens from the Volga in Russia, all from the study of Perrot-Minnot et al. (2018) and also assigned to *P. laevis*.

The second main clade (II), here designated as "*P. laevis*-clade" is divided into two closely related subclades, one comprising GenBank sequences assigned to *P. laevis* by Perrot-Minnot (2004) and Moret et al. (2007) and originating from Hungary, France and the Czech Republic (*P. laevis 1*). The other clade comprises sequences of morphologically identified *P. laevis* of the present study as well as sequences of *P. laevis* from NCBI GenBank from mainly P.-C. Europe (*P. laevis 2*).

3.2.2. Genetic p-distances

The intraspecific genetic *p*-distance was lowest in *P. bosniacus* with a mean distance of 0.5% and a maximal distance of 2.4%. The species *P. laevis* (both subclades) showed a mean genetic *p*-distance of 2% (maximum 3.5%). Average intraspecific genetic distance in *P. tereticollis* was 1.8% and the highest one 3.8%. Sequence divergence between *P. bosniacus* and *P. laevis* ranged from 8.2% to 10.9% with an overall genetic distance of 10.2%. The observed genetic *p*-distances were highest between *P. bosniacus* and *P. tereticollis*, ranging from 22.2% to 23.5% (22.7%). The distances between *P. tereticollis* and *P. laevis* ranged from 18.9% to 20.9% with an average of 19.7%.

Table 3
Genetic diversity parameters and distances (*p*-distances in %) for the *COI* dataset.

species	no. sequences	no. haplotypes	haplotype div. (Hd)	nucleotide div. (π)	Ø distances	max. distances	*P. tereticollis*	*P. laevis*
P. tereticollis	73	37	0.972	0.018	1.8	3.8		
P. laevis	87	35	0.949	0.019	2	3.5	19.7 (18.9–20.9)	
P. bosniacus	92	43	0.868	0.005	0.5	2.4	22.7 (22.2–23.5)	10.2 (8.2–10.9)

3.2.3. Haplotype networks and mitochondrial diversity

We calculated haplotypes of the three clades including sequences from the present study.

Among 92 sequences of *P. bosniacus* 43 haplotypes were found (Fig. 3 B; Table 3). The median-joining network showed a radial structure with a most frequently observed haplotype shared by 33 individuals, i.e. containing most of the specimens of this study as well as NCBI GenBank sequences from different eastern European countries and from the Upper Rhine in France (Fig. 3 B). The low nucleotide diversity (π = 0.005) is partly due to the high frequency of one very common haplotype (Table 3). Furthermore, several haplotypes connected by short branches with this widespread haplotype were found. The comparatively high haplotype diversity (HD = 0.868) is due to the high number of haplotypes (Table 3) shared by only one or two individuals. Only two haplotypes of GenBank sequences from Bulgaria, Hungary and Austria were separated by eight mutation steps from the main haplo-group.

The haplotype network of *P. laevis* shows two haplogroups separated by 13 mutation steps (Fig. 3 C). Among the sequences from *P. laevis* generated in this study only two haplotypes were found. They are closely related to the main haplotype *P. laevis 2*, which is shared by specimens from Eastern Carpathian and Northern Aegean (Fig. 3 C). The other haplogroup, *P. laevis 1*, is represented by a Western Carpathian-Pannonian-Western European sublineage. The network shows a high haplotype diversity (Hd = 0.949) but a considerably low nucleotide diversity (π = 0.019) (Table 3).

Pomphorhynchus tereticollis (Fig. 3 D) showed a high intraspecific variation with a high haplotype diversity (Hd = 0.972) but a considerably low nucleotide diversity (π = 0.018) (Table 3). The network consists of 37 haplotypes and it is roughly divided into four clusters of haplotypes, reflecting a geographical structuring: the first includes specimens from France (Fig. 3 D1), the second contains specimens from Central and Northern Europe infecting mostly marine fishes (Fig. 3 D2), and the third cluster includes animals from several European countries including Austrian samples of the present study (Fig. 3 D3). Within this cluster quite high distances were found between specimens from France and Slovakia. The fourth cluster comprises only Austrian specimens (Fig. 3 D4). Remarkably, specimens from the same geographic region (obtained from rivers in Germany, France and Austria) are present in two clusters, but considerably high distances were observed between these clusters (Fig. 3 D1, D3, D4). Hereby, the specimens of this study were divided into two separate clades, although the collection locality was the same.

4. Discussion

In the present study, we report three species of the genus *Pomphorhynchus* in Austria: *P. tereticollis, P. laevis* and *P. bosniacus*. We provide morphological descriptions and documentation of the relevant structures and we determined DNA barcodes of reference specimens. While *P. tereticollis* and *P. laevis* were mostly found to infect fish in rivers in Styria, *P. bosniacus* was the predominant species in the Danube. It was the first evidence of the occurrence of *P. bosniacus* in Austria; in fact, it was never reported for Central and Western Europe before. To the best of our knowledge there are no published studies documenting the occurrence of *P. tereticollis* in Austria.

The presence of *P. bosniacus* in Austria was revealed in the Danube, in this case in the area of Freudenau in Vienna. Although the morphological appearance of *P. bosniacus* is rather reminiscent to *P. tereticollis* (cf. Fig. 2 A, B, D, E), the genetic analysis revealed a genetic *p*-distance of 22.7% to *P. tereticollis* but only 10.2% to *P. laevis* (Table 3) even though *P. bosniacus* is morphologically considerably distinct from *P. laevis*. However, sequences which correspond to the *mt* lineage determined as *P. bosniacus* in the present study, were published in NCBI GenBank as *P. laevis* (David et al., 2017; Perrot-Minnot et al., 2018). Since there is no information given by the authors about the

morphology of their examined specimens, we cannot conclude if morphological variations exist between our specimens and those examined by David et al. (2017) and Perrot-Minnot et al. (2018). Intraspecific morphological variations are present in *P. laevis* (Brown, 1987) in the number of hooks per row (from 12 to 13) and in the number of longitudinal hook rows, which can vary from 18 to 20 (Meyer, 1933; Petrochenko, 1956; Kiskároly and Čanković, 1967). In the present analysis we always counted 12 hooks per row (in four individuals). The longitudinal rows were counted in the 3-D reconstruction in one individual. Therefore, our data cannot contribute to the question on intraspecific variation. The furcation of the hook root basis as described by (Meyer, 1933) was not confirmed by the 3-D reconstruction, yet only a single individual was analyzed. It remains to be investigated whether the furcation is a misinterpretation of whole-mount preparations and if the matrix between the outer parts of the root is generally present. Nevertheless, despite the different genetical assignments between our study and the ones of David et al. (2017) and Perrot-Minnot et al. (2018), the morphological traits determined in this study may justify the assignment to *P. bosniacus*. *Pomphorhynchus laevis* does not exhibit the basal hooks attached in the center of the hook roots, the last circle of hooks is located on the posterior part of the proboscis of *P. laevis* (on the anterior part of the bulb in *P. bosniacus*), and it possesses a higher number of longitudinal hook rows than *P. bosniacus*. Thus, the distinct morphological traits of *P. bosniacus* observed in specimens of this study do not suggest a distinct *mt* lineage of *P. laevis*. Although some variations in the measurements between our specimens and the one in the original description of Kiskároly and Čanković (1967) are given, distinct traits like the attachment of the basal hooks in the center of the roots, the arrangement of the hooks, the stout appearance of hooks number four and five as well as the variations in the shape of the bulb are present in the specimens of this study and are in agreement with the description of *P. bosniacus* (Kiskároly and Čanković, 1967; Moravec, 2004). The variations in the shape of the bulb can be considered as intraspecific variation known in some species of *Pomphorhynchus*, which makes the importance of the bulb for species identification questionable (Li et al., 2017). Unfortunately, an examination of the original material of *P. bosniacus* as further validation of this assignment was impossible, since the original material of *P. bosniacus* was not available at the Veterinarian institute of Sarajevo, Bosnia Herzegowina, where it was supposed to be deposited.

It is difficult to conclude when *P. bosniacus* started to occur in Austria, due to its sparse, previous documented distribution, which ranges only from lakes and rivers in the Balkans (Kakacheva-Avramova, 1973; Hristovski, 1999; Moravec, 2004; Cakic et al., 2008). David et al. (2017), who found the *mt* lineage of *P. bosniacus* in the Upper Rhine, explained its occurrence with the invasion of the round goby *Neogobius melanostomus* from P.-C. Europe. This could be an indication of a co-invasion of host and parasite, as proposed by David et al. (2017), who described this lineage as an exotic lineage of *P. laevis*. This would agree with the rapid expansion of *N. melanostomus*, which was first reported in Austria in the year 2000 (Wiesner et al., 2000). Also, invasive gammarids from the P.-C. region like *Dikerogammarus villosus* (Sowinsky, 1894) (Gammaridae) were associated with the expansion of species of *Pomphorhynchus* by serving as intermediate hosts (Emde et al., 2012; Hohenadler et al., 2017). Since 1989, *D. villosus* invaded the Danube in Austria (Nesemann et al., 1995) and its mass-occurrences lead to a decline of the native *Gammarus pulex*, which is known as intermediate host of *P. laevis* (Emde et al., 2012). Based on the present data, the so called "*P. laevis* exotic lineage" must be assigned to *P. bosniacus* and following up the assumption of David et al. (2017), this putative invader of Central and Western Europe would have been *P. bosniacus* and not *P. laevis*. Perrot-Minnot et al. (2018) support this assumption of a co-invasion due to star-like haplotype network of this species, which might be an indication of a rapid expansion. However, despite this very common, widespread haplotype, the network of *P. bosniacus* shows a very high haplotype diversity which contradicts the

assumption of a relatively recent population expansion (Fig. 3 B). Specimens obtained from the Upper Rhine show a very high diversity, but non-native gobies from P.-C. Europe invaded the Upper Rhine only recently within the years 2007–2012 (Manné et al., 2013). This implies that *P. bosniacus* might have a longer history in the Rhine and the Danube and probably was overlooked so far. However, Perrot-Minnot et al. (2018) suggest a diversification in the *mt*-lineage of *P. bosniacus* during the Pleistocene (around 1.27 Myr ago) within the northern Black Sea-Caspian Sea region. The establishment of *P. bosniacus* in the Danube might have occurred during the end of the Pleistocene by the expansion of the main host *B. barbus* from its Black Sea refugium (Kotlik et al., 2004). *Barbus barbus* reached Western Europe during the last interglacial (~130 000–115 000 years ago) with a glacial refugium in southern France from where it expanded into Western European rivers (Kotlik and Berrebi, 2001; Kotlik et al., 2004). This phylogeography of *B. barbus* might be an explanation for the high haplotype diversity in *P. bosniacus* within the population of the Upper Rhine.

According to the literature *P. laevis* should be the predominant species of the genus in the Danube (Kritscher, 1985; Moravec et al., 1997; Laimgruber et al., 2005) and it was long time known as the most abundant species of *Pomphorhynchus* in Europe (e.g. Kennedy, 2006; Špakulová et al., 2011). However, *P. laevis* was found in this study only in two rivers (Mur and Sulm) in Styria in small numbers and in larval or juvenile life-stages (Table 1, Fig. 1). These specimens belong to a lineage first described for the river Sava in Croatia (Vardić Smrzlić et al., 2015). Additional sequences from other Eastern European countries belonging to this lineage were published by Perrot-Minnot et al. (2018). The Eastern European origin of this lineage is also reflected in the haplotype-network and it shows a high intraspecific variability (Fig. 3 C). The establishment of a population of this Eastern lineage in the rivers Mur and Sulm might have occurred via the Mur-Drau-Danube riverine landscape (Fig. 1). In the Austrian sample the subclade *P. laevis 1* was not detected. It would be interesting to obtain morphological data from individuals representing *P. laevis 1* to assess their taxonomic status. The fact that we did not detect *P. laevis* in the Danube in Austria can be explained in two ways: (1) *P. bosniacus* outcompeted *P. laevis*. This scenario is supported by the work of Perrot-Minnot et al. (2018), who found *P. laevis* mostly in the tributaries of the Danube, while the lineage representing *P. bosniacus* was the dominant species in the Danube. (2) *P. bosniacus* was repeatedly misidentified as *P. laevis*. The latter explanation is supported by the fact that specimens from a large geographic range were assigned to *P. laevis* in the past and recently proved to represent genetically highly distinct lineages (Perrot-Minnot et al., 2018).

In general, whether and which of the distinct lineages (Fig. 3) formerly assigned to *P. laevis* represent distinct (cryptic) species remains to be investigated. Our combined morphological and genetic data provide evidence at least for one of these lineages, namely *P. bosniacus*.

Also, the species *P. tereticollis* showed a high prevalence in the river Mur infecting a wide range of cyprinid and salmonid fishes (Table 1) suggesting that its distribution is much wider than previously assumed. This assumption is supported by the haplotype network which shows a high intraspecific variability between the Austrian *P. tereticollis* and *P. tereticollis* specimens elsewhere in Europe (Fig. 3 D). An explanation for the underestimated distribution of *P. tereticollis* might be its morphological similarity to *P. laevis* (Špakulová et al., 2011; Hohenadler et al., 2017). Earlier misidentifications of the latter species and synonymy of *P. laevis* and *P. tereticollis* may have caused false conclusions concerning their distribution (Hohenadler et al., 2017). It is not resolved yet if the two species co-occur in the same locality, although such a co-occurrence seems doubtful (Emde et al., 2012; Hohenadler et al., 2017). Hohenadler et al. (2017) mentioned a decline of *P. tereticollis* due to *P. laevis* in Switzerland. Indeed, a former co-existence in the River Rhine was reported for the years 2003 and 2004 resulting in the displacement of *P. tereticollis* by *P. laevis* (Hohenadler et al., 2017). We, however, found both species in the same localities (rivers Mur and Sulm) in

Styria, an evidence that the two species can co-exist (Fig. 1). To determine the future trend of this co-existence, a long-term investigation would be necessary as conducted in the Rhine in Germany. Furthermore, the role of *P. bosniacus* needs to be clarified because it is unknown since when it occurred in Austria and how the parasite-fauna changed in the Danube since its appearance. The fact that we found one individual of *P. tereticollis* in the Danube might be an indication that the species occurs in the Danube, at least in small numbers. This should be clarified by a broader survey. Also, it cannot be reconstructed if *P. tereticollis* also invaded Austria recently or if it occurred there in former times and was just misidentified as *P. laevis* in previous studies. Perrot-Minnot et al. (2018) assumed an ancestral population of *P. tereticollis* in the Rhône drainage (Fig. 3 D1) from where the species should have dispersed northwards to the North Sea and the Baltic Sea (Fig. 3 D2) and eastward to the Rhine and Carpathians (Fig. 3 D3). Prior to our study, this species was not recorded in Austria at all. In the present study we found a lineage of *P. tereticollis*, distinct to known lineages. The origin of this lineage is yet unknown. More biogeographical research and molecular dating with more samples would be necessary to address the question since when *P. tereticollis* appeared in Austria. For further validation, museum collections of parasitic helminths can be examined, although the fixation in formaldehyde might inhibit molecular genetic methods (Zimmermann et al., 2008). However, the integrative approach including additional sectioning and reconstructing of the hooks on the proboscis, highlights the benefits of such methods in the identification of Acanthocephala. For example, species-specific traits like the extensions on the basal hooks in *P. tereticollis* which are only visible in living specimens were also visible in the confocal laser scans and in the 3-D reconstruction of fixed material. This enables a morphological identification also in fixed material.

It is important to note that only juveniles and cystacanth stages of *P. laevis* and *P. tereticollis* obtained from the rivers Mur and Sulm were found. Especially the wide host range of *P. tereticollis* observed in this study might be an indication that some of these fish species might be paratenic hosts. It should be emphasized that we could not find any adult specimens of *P. tereticollis* or *P. laevis* in our study despite the wide range of fish species examined. Therefore, it is still necessary to investigate whether these life stages are part of viable life cycles or just dead-end infections (Kennedy, 2006; Médoc et al., 2011). Parateny has important values for the life cycle and ecology of the parasite, despite the fact that it cannot complete its life cycle within the paratenic host (Euzeby, 1997; Kennedy, 2006). For example, due to parateny the parasite can also survive in absence of a suitable host or, due to the fact that a paratenic host can be infected several times by infective parasite stages, high concentrations can lead to a heavy infection of the final host (Euzeby, 1997; Kennedy, 2006). However, any unsuitable host representing a dead-end would lead to a decline in the parasite population (David et al., 2017). Therefore, further investigations regarding the host specificity of *Pomphorhynchus* spp. are necessary.

5. Conclusions

We established the occurrence of three species of the genus *Pomphorhynchus* in Austrian waters by using an integrative-taxonomic approach. This approach enabled a thorough examination of *Pomphorhynchus* species and we were able to identify the species *P. bosniacus,* the distribution of which throughout Europe probably might have been misinterpreted in previous studies. The high intraspecific variability visible in the haplotype network contradicts a recent population expansion towards Western Europe of this species. Furthermore, there is still a lack of knowledge since when the three species occur in Austria and how this parasite-composition changed during time. Misidentifications in prior times might have led to false conclusions regarding the distribution of *Pomphorhynchus* spp. Also, the role of paratenic hosts in the life cycle of *P. tereticollis* and *P. laevis* still needs further investigations. The importance of integrative methods and the

establishment of a reliable database of DNA sequences like BOLD and the Austrian Barcode of Life (ABOL) initiative to identify species of *Pomphorhynchus* became evident, since the probability of cryptic species within the species *P. laevis* is quite likely. Therefore, a revision of the genus in Europe using integrative methods would be recommended.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.01.009.

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