Research Article

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Unravelling the hidden biodiversity – the establishment of DNA barcodes of fish-parasitizing Acanthocephala Koehlreuther, 1771 in view of taxonomic misidentifications, intraspecific variability and possible cryptic species

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Abstract

Acanthocephalans are obligate parasites of vertebrates, mostly of fish. There is limited knowledge about the diversity of fish-parasitizing Acanthocephala in Austria. Seven determined species and an undetermined species are recorded for Austrian waters. Morphological identification of acanthocephalans remains challenging due to their sparse morphological characters and their high intraspecific variations. DNA barcoding is an effective tool for taxonomic assignment at the species level. In this study, we provide new DNA barcoding data for three genera of Acanthocephala (*Pomphorhynchus* Monticelli, 1905, *Echinorhynchus* Zoega in Müller, 1776 and *Acanthocephalus* Koelreuter, 1771) obtained from different fish species in Austria and provide an important contribution to acanthocephalan taxonomy and distribution in Austrian fish. Nevertheless, the taxonomic assignment of one species must remain open. We found indications for cryptic species within *Echinorhynchus cinctulus* Porta, 1905. Our study underlines the difficulties in processing reliable DNA barcodes and highlights the importance of the establishment of such DNA barcodes to overcome these. To achieve this goal, it is necessary to collect and compare material across Europe allowing a comprehensive revision of the phylum in Europe.

Introduction

The phylum Acanthocephala represents obligatory parasites infesting intestines of vertebrates (Kennedy, 2006). So far, 22 fish-parasitizing species belonging to the families Echinorhynchidae (mostly fish-parasitizing) and Pomphorhynchidae (exclusively fish-parasitizing) are reported for Europe (Gibson *et al.*, 2014). Due to their ambiguous morphological characters (Kennedy, 2006), individual variability (Cleave, 1952) and the potential occurrence of cryptic species (e.g. Steinauer *et al.*, 2007; Zittel *et al.*, 2018), taxonomic identification of acanthocephalans remains challenging. DNA barcoding has proven as an important tool for species identification, as seen in recent studies on the genus *Pomphorhynchus* that revealed previous contradictory determinations with a reliable DNA barcoding library (e.g. David *et al.*, 2017; Perrot-Minnot *et al.*, 2018; Reier *et al.*, 2019). Such inconsistencies are often attributed to strain formations in parasitic taxa due to different ecological demands and/or adaptations to host species (e.g. O'Mahony *et al.*, 2004; Steinauer *et al.*, 2007; Perrot-Minnot *et al.*, 2018; Grabner *et al.*, 2020). However, DNA barcode sequences of acanthocephalans in Europe are so far rare and the standard mitochondrial barcoding marker gene *cytochrome c oxidase subunit 1* (*COI*) was rarely applied in the identification of this phylum in Europe. Most *COI* sequences of Acanthocephala were processed to investigate phylogenetic relationships. However, morphological data belonging to these sequences are lacking.

According to Kritscher (1985), who performed the latest classification of Acanthocephala in Austria 35 years ago, three classes, nine families, 18 genera and 37 species of Acanthocephala were recorded for Austria. Thereof, five species of four genera were recognized as fish parasites: *Neoechinorhynchus rutili* (Müller, 1780) a member of the class Eoacanthocephala, and *Acanthocephalus anguillae* (Müller, 1780), *Acanthocephalus lucii* (Müller, 1776), *Echinorhynchus truttae* Schrank, 1788 and *Pomphorhynchus laevis* (Müller, 1776), all belonging to the class Palaeacanthocephala (Kritscher, 1985). In addition, Rydlo (1990) reported *Pseudoechinorhynchus clavula* (Dujardin, 1845) from *Lota lota* (L.) obtained from different waters in Austria, but mentioned that according to a personal comment of Prof O. N. Bauer from Russia it might be assigned to *Echinorhynchus borealis* Linstow, 1901 (syn. *Echinorhynchus cinctulus* Porta, 1905).

So far, there is only scarce data regarding the distribution and occurrence of fish-parasitizing Acanthocephala in Austria. In the course of the Austrian Barcode of Life (ABOL) initiative we performed an investigation of acanthocephalans from a selection of Austrian freshwater fish and identified them morphologically, followed by molecular genetic analyses. First results of that investigation were published by Reier et al. (2019) and focused on the genus Pomphorhynchus. That study revealed the occurrence of two additional species in Austria by combining morphological and molecular genetic methods: Pomphorhynchus tereticollis (Rudolphi, 1809) and Pomphorhynchus bosniacus Kiskároly & Čanković, 1967. Here we present data on all fish-parasitizing Acanthocephala detected during the ABOL survey. The sequences processed in this study were combined with those from our previous study on Pomphorhynchus (Reier et al., 2019) and compared to other published sequences from GenBank.

The aims of this study were 2-fold: (1) we aimed to get new insights on taxa occurring in Austrian freshwater fish, and to complement the recent knowledge about the occurrence and host range of acanthocephalans in Austria (and thus Central Europe). (2) We established reliable DNA barcodes for the ABOL and Barcode of Life (BOLD) databases, which can be applied in the identification of acanthocephalans. The DNA barcodes generated should simplify future identifications in Acanthocephala.

Materials and methods

Host and parasite sampling

In this study (combined with our previous study), 203 fish specimens or fish intestines from different rivers and lakes of Styria, Burgenland, Carinthia, Vienna and Lower Austria were provided by various working groups and persons in Austria (see Acknowledgements). Identifications of fish specimens were performed by the providers. Altogether eight fish species were included in this study (Table 1). Forty-eight of the dissected fish or intestines contained 302 helminths (242 Acanthocephala, 11 Nematoda, 3 Cestoda and 46 Trematoda) which were preserved in 80% ethanol and stored at 4°C. The current study provides an overview on acanthocephalans, while all other helminths will be presented elsewhere. Sixty-four specimens of the genus Pomphorhynchus were processed in our previous study morphologically and/or genetically. In this study additional 37 specimens of Acanthocephala were investigated morphologically and genetically. The taxa recorded in the course of this study as well as the host species are summarized in Table 1. All analysed specimens are deposited in the collection Evertebrata Varia of the Natural History Museum Vienna (museum collection numbers are given in Table 1). Table S1 of Supporting information lists all analysed specimens in the former and current study.

Morphological methods

Specimens were identified morphologically according to species-specific morphological characters mentioned in the literature (Lühe, 1911; Meyer, 1933; Petrochenko, 1956; Golvan, 1969; Grabda-Kazubska and Ejsymont, 1969; Špakulová et al., 2011; Reier et al., 2019). In Pomphorhynchus, morphological assignment at the species level is challenging due to morphological similarity and faint differentiating traits, especially regarding the species P. tereticollis, P. laevis and P. bosniacus (Špakulová et al., 2011; Hohenadler et al., 2017; Reier et al., 2019). Reier et al. (2019) did thorough morphological investigations [including confocal laser scanning microscopy (CLSM) and 3-D reconstruction] and had already established reliable DNA barcodes of those

species. Therefore, the taxonomic assignment for Austrian Pomphorhynchus species of the current study relies on those reference sequences.

Since a short-term treatment with glycerol has been shown to have no negative impact on DNA quality (Reier et al., 2020), specimens were cleared in a mixture of glycerol and 80% ethanol (1:1) and incubated overnight at 38°C until ethanol was evaporated. Subsequently, specimens were transferred to a microscope slide with pure glycerol as mounting medium for investigations under the light microscope. Microphotographs were taken with a Nikon Eclipse Ni-U microscope equipped with a Nikon DS-Ri2 microscope camera and measurements were performed in NIS Elements software (Nikon Instruments Europe BV, Amsterdam, Netherlands). Length and width of body trunk, proboscis, proboscis receptaculum, lemnisci and testes as well as hook length were measured for specimens of Echinorhynchus cinctulus.

Analysis of autofluorescence spectra of different excitation wavelengths was conducted for E. cinctulus with the CLSM Leica TCS SP5 II (Leica Microsystems, Wetzlar, Germany). Image stacks were merged into maximum intensity projection images and further imported and processed in FIJI (Schindelin et al., 2012), or by volume rendering using AMIRA 6.11 (FEI; Hillsboro, Oregon, USA). The CLSM stacks are deposited on the Phaidra repository of the University of Vienna (Table S2 of Supporting information).

Molecular genetic methods

DNA extraction was carried out in a clean room using the QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). For DNA barcoding a partial sequence of the mitochondrial cytochrome c oxidase unit 1 gene (COI) was used. Primer pairs for different taxa and the corresponding annealing temperatures as well as amplicon sizes are given in Table 2. Details of DNA extraction and polymerase chain reaction (PCR) amplification are the same as described in Reier et al. (2019). The PCR products were sequenced (both directions) by Microsynth (Balgach, Switzerland) using the PCR primers if not stated otherwise in Table 2.

Sequences were processed in Geneious 2.10.3 (https://www.geneious.com). For understanding the position of examined genera among already published sequences we included COI sequences from GenBank of the following genera into our final alignment: Pomphorhynchus spp. [MK612497–MK612545 (Reier et al., 2019)], Acanthocephalus spp. [KP261016 (Wayland et al., 2015), AM039836, AM039837, AM039864–AM039866 (Benesh et al., 2006), DQ089718 (García-Varela and Nadler, 2006), MN416027–MN416030 (Amin et al., 2019)] and Echinorhynchus spp. [KP261013, KP261014, KP261017–KP261019 (Wayland et al., 2015), AY218095 (Giribet et al., 2004), DQ089710 (García-Varela and Nadler, 2006)]. The rotifer Lecane bulla (Gosse, 1851) (HQ944350) was used as outgroup to root the tree. The final alignment contained 102 sequences and was trimmed to 544 bp due to different lengths of sequences. A neighbour joining (NJ) tree was calculated in MEGA 10.0.5 (Kumar et al., 2018). FigTree 1.4.3 (http://tree.bio.ed.ac.uk) was used for graphical processing of the tree and InkScape 0.92 (https://inkscape.org) for the final layout. Inter- and intraspecific P-distances (pairwise deletion) were calculated using MEGA 10.0.5 (Kumar et al., 2018).

Median joining (MJ) haplotype networks (Bandelt et al., 1999) were calculated for the species P. tereticollis, P. bosniacus, E. cinctulus, A. anguillae sensu lato (according to Amin et al., 2019) and A. lucii using PopART 1.7 software (http://www.popart.otago.ac.nz). For A. lucii, to allow inclusion of shorter GenBank sequences

Table 1. List of all genetically analysed specimens from this study

Lab	Museum	BOLD	GenBank	Host species	Waterbody
Pomphorhynchus tereticollis					
BF-P-001	21278	ACANT082-20	MT682954	*Oncorhynchus mykiss*	Stattegg (fish pond), S
BF-P-002	21279	ACANT083-20	MT682955	*O. mykiss*	Stattegg (fish pond), S
15042016-15A	21280	ACANT084-20	MT682956	*Barbatula barbatula*	River Leitha, B
Pomphorhynchus bosniacus					
BF-P-003	21281	ACANT085-20	MT682953	*O. mykiss*	Stattegg (fish pond), S
BF-P-004	21282	ACANT086-20	MT682952	*O. mykiss*	Stattegg (fish pond), S
Echinorhynchus cinctulus					
Fish26-2	21283	ACANT050-20	MT682920	*Zingel streber*	River Mur, S
Fish45-3	21284/1	ACANT051-20	MT682921	*Lota lota*	River Mur, S
Fish45-4	21284/2	ACANT052-20	MT682922	*L. lota*	River Mur, S
Fish45-6	21284/3	ACANT053-20	MT682923	*L. lota*	River Mur, S
Fish45-7	21284/4	ACANT054-20	MT682924	*L. lota*	River Mur, S
BR21-1	21285	ACANT055-20	MT682925	*Cottus gobio*	Lunz (brook), LA
KBO25-1	21286	ACANT056-20	MT682926	*C. gobio*	Lunz (brook), LA
SR24-1	21287/1	ACANT057-20	MT682927	*C. gobio*	Lunz (brook), LA
SR24-2	21287/2	ACANT058-20	MT682928	*C. gobio*	Lunz (brook), LA
SR-12-1	21288	ACANT059-20	MT682929	*C. gobio*	Lunz (brook), LA
YGL-16-1	21289	ACANT060-20	MT682930	*C. gobio*	Lunz (brook), LA
Acanthocephalus anguillae					
AAL9-1A	21290	ACANT061-20	MT682931	*Anguilla anguilla*	Lake Neusiedl, B
AAL15-A	21291	ACANT062-20	MT682932	*A. anguilla*	Lake Neusiedl, B
AAL20A	21292	ACANT063-20	MT682933	*A. anguilla*	Lake Neusiedl, B
AAL21-3	21293	ACANT064-20	MT682934	*A. anguilla*	Lake Neusiedl, B
Acanthocephalus sp.					
AAL23A	21294	ACANT065-20	MT682935	*A. anguilla*	Lake Neusiedl, B
Acanthocephalus lucii					
BA2-3	21297/1	ACANT066-20	MT682936	*Perca fluviatilis*	Waidhofen/T. (fish pond), LA
BA1-1	21295	ACANT067-20	MT682937	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
A16022016	21296/1	ACANT068-20	MT682938	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
A16022016-2A	21296/2	ACANT069-20	MT682939	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
FI28-1	21304	ACANT070-20	MT682940	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA2-4	21297/2	ACANT073-20	MT682943	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA2-6	21297/4	ACANT074-20	MT682944	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA2-5	21297/3	ACANT075-20	MT682945	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA6-1	21298	ACANT076-20	MT682946	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA13-1	21299	ACANT077-20	MT682947	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
BA18-1	21300	ACANT078-20	MT682948	*Gymnocephalus cernua*	Waidhofen/T. (fish pond), LA
FI16-1	21301	ACANT079-20	MT682949	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
FI17-1	21302/1	ACANT080-20	MT682950	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
FI17-2	21302/2	ACANT081-20	MT682951	*P. fluviatilis*	Waidhofen/T. (fish pond), LA
AAL-N19-1	21303/1	ACANT071-20	MT682941	*A. anguilla*	Lake Neusiedl, B
AAL-N19-3	21303/2	ACANT072-20	MT682942	*A. anguilla*	Lake Neusiedl, B

S, Styria; B, Burgenland; LA, Lower Austria.
IDs for lab, museum, BOLD and GenBank are given.

Table 2. Primer sets used in this study

Primer name	DNA sequence (5′–3′)	Primer annealing temperature (°C)	Estimated size (bp)	Primer source
COI amplification Acanthocephala taxa (exception *A. lucii*)				
H14AcanCOIFw1	TTCTACAAATCATAARGATATYGG	48	~650	Reier *et al.* (2019)
H14AcanCOIRv2	AAAATATAMACTTCAGGATGACCAAA			Reier *et al.* (2019)
COI amplification *A. lucii*				
Acan-3+	TTAGTGGGCGTATCATTGAGT	56	~540	This study
Acan-4−	ACAAGATACTTTGACCCATGCGGA			This study
Sequencing primers *P. tereticollis*				
P_tere_COI_fw	TTGATGGGGTTTTCTATAAGG	50	~650	This study
H14AcanCOIRv2	AAAATATAMACTTCAGGATGACCAAA			Reier *et al.* (2019)
Sequencing primers *P. bosniacus*				
P_bos_COI_fw	CTAATGGGGTTTTCTATAAGG	50	~650	This study
H14AcanCOIRv2	AAAATATAMACTTCAGGATGACCAAA			Reier *et al.* (2019)

from Finland [AM039878, AM039880–AM039884 (Benesh *et al.*, 2006)], the alignment was trimmed to 428 bp.

Results

Morphological determination

Acanthocephalans of three different fish species from Lower Austria and Burgenland were clearly assigned to the genus *Acanthocephalus*. Two species were differentiated morphologically as *A. lucii* and *A. anguillae* according to characters described in the literature (Meyer, 1933; Petrochenko, 1956; Golvan, 1969). Four specimens of *A. anguillae* [from *Anguilla anguilla* (L.)] were juvenile and exhibited a wrinkled body form. Nevertheless, species determination was possible based on the characteristic lateral extensions on the hooks of the proboscis and the constant number of six large hooks in the lateral rows. Only the last hook was considerably smaller without any extensions.

Sixteen specimens [obtained from *A. anguilla*, *Gymnocephalus cernua* (L.) and *Perca fluviatilis* (L.)] were determined as *A. lucii*. Morphological determination was straightforward since the analysed specimens were adults and corresponded to the descriptions and measurements known from the above-mentioned literature: we counted eight hooks in a row with wing-like shaped hook roots as shown in Meyer (1933) as a typical character of *A. lucii*. Also, the hooks were longer than the hook roots and only the last row of hooks exhibited considerably smaller hooks.

One additional specimen (from *A. anguilla*) could not be identified due to its bad condition. It had a length of 5.15 mm and the proboscis was invaginated.

Eleven specimens were morphologically assigned to the genus *Echinorhynchus*. Five specimens from Styria obtained from *L. lota* and *Zingel streber* (Siebold 1863) were juvenile with an invaginated proboscis, therefore a thorough morphological investigation was impossible. The six specimens from Lower Austria obtained from *Cottus gobio* (L.) were adults (Fig. 1). Measurements of length and width of body trunk, proboscis, proboscis receptaculum, lemnisci and of testes of four males and seven females are given in Table 3. The appearance of the hooks was mostly uniform with simple hook roots without appendices. Furthermore, confocal laser scans were processed for two specimens (even a juvenile from *L. lota*) and the invaginated hooks and hook rows were counted. In these scans 12–14 hooks per row became apparent. The basal hooks were considerably smaller (26–34 μm) than the largest hooks in the centre of the proboscis (53–58 μm).

Furthermore, we counted an additional rudimental hook on the ventral surface of the proboscis with a length of 14–15 μm. All measurements were in concordance with the thorough analysis of *Echinorhynchus borealis* Linstow, 1901 by Grabda-Kazubska and Ejsymont (1969). This species was later synonymized with *Echinorhynchus cinctulus* Porta, 1905, and therefore, we assigned the specimens (including the juveniles) to *E. cinctulus*.

Five specimens were morphologically assigned to the genus *Pomphorhynchus* due to the genus-specific traits like the long neck and the bulb. Morphological assignment at the species level is rather challenging due to only faint distinguishing characteristics. Species assignment was performed according to Reier *et al.* (2019) combining morphological investigation and DNA barcodes. Three out of the five specimens were assigned to *P. tereticollis* [two from *Oncorhynchus mykiss* (Walbaum, 1792) and one from *Barbatula barbatula* (L.)], and two were determined as *P. bosniacus* (both from *O. mykiss*).

DNA analysis

The *COI* sequences of the 36 individuals analysed in the current study were combined with sequences from our earlier study and other sequences from GenBank. The final alignment of 544 bp comprised *COI* sequences of 102 specimens. The distances between lineages are illustrated in a *NJ* tree (Fig. 2). Nearly all major nodes had high bootstrap support. With one exception (one sequence from GenBank of *Echinorhynchus salmonis* appeared as a quite distinct lineage), genera were supported by high bootstrap values, too. While the *NJ* tree provides an overview, the sequences of the three genera detected in the current study (Fig. 2A, marked with different colours) are hereafter described in detail based on the corresponding MJ networks.

Two main clades and two lineages represented by one GenBank sequence each (one *A. clavula*, the other one not determined at the species level) were distinguished for the genus *Acanthocephalus* in the *NJ* tree (Fig. 2). The individuals analysed in the current study are found in the two main clades shown in detail in the networks (Fig. 2E and F). Interestingly, *A. anguillae* is found in both main clades.

The MJ network reveals a high variation for *A. anguillae* sensu lato (Fig. 2E). We identified six haplotypes. The network is star-like with the haplotype of one *A. anguillae* individual of this study in the centre (Fig. 2C). The other haplotype of *A. anguillae* detected in this study (shared by two individuals) is separated by two mutation steps. Sequences of *A. anguillae* from

Fig. 1. *Echinorhynchus cinctulus* from *C. gobio*: (A) male and (B) female. Scale bar: 500 µm

Germany are found in two haplotypes, separated from the central haplotype by three and one mutation steps, respectively (mean *p*-distance 0.5%, Table 4). Eight mutation steps (1.8%) separate the central haplotype from a sequence from GenBank assigned to the recently described subspecies *Acanthocephalus anguillae balkanicus* (Amin *et al.*, 2019). Finally, a GenBank sequence of *A. dirus* is separated by seven mutation steps (mean genetic *p*-distance of 1.6%).

For the *MJ* network of the second main clade we included shorter GenBank sequences of *A. lucii* from Finland into the alignment, thus the network shows one more haplogroup (A2) compared to the tree (Fig. 2D). Altogether 13 haplotypes were found. Haplogroup A1 contains 16 sequences of this study that were morphologically assigned to *A. lucii*. It is separated by seven mutation steps (2.9%) from the Finnish *A. lucii* haplogroup (A2). The haplogroup comprising GenBank sequences assigned to *A. lucii* from the UK (A3) and a haplotype assigned to *A. anguillae* from UK (A4) is separated by 20 mutation steps from subclade A2 and by 27 steps from subclade A1. Mean *p*-distance between subclades A1 and A3 was 2.6% and between subclades A1 and A4 6.3%, while the mean *p*-distance between A3 and A4 was only 1.4% (Table 4).

One single sequence belonging to a specimen of this study (*Acanthocephalus* sp.) represented a distinct lineage not closely related to any clade of *Acanthocephalus* of the current study or GenBank, respectively (Fig. 2A).

Specimens of the current study and determined as *E. cinctulus* are found within one clade (E) which is divided into two subclades (E1 and E2). The sister group of this clade is a published sequence of *E. cinctulus* (KP261014, Table 4) which, however, is very distantly related (mean *p*-distance 20%) to the specimens analysed in the current study. Clade E can be divided into two subclades, one (subclade E1) containing specimens from Styria obtained from *L. lota* and *Z. streber*, and the other one (subclade E2) including specimens from Lower Austria from *C. gobio*. The haplotype network shows the relationships between the six haplotypes of clade E. The two haplogroups (E1 and E2) are separated by 12 mutation steps (mean distance 2.9%, Table 5). Mean genetic distances within the two haplogroups are given in Table 5.

The other lineages of *Echinorhynchus* are formed by sequences from GenBank: besides, the highly distant *E. cinctulus* sequence, there is one clade comprising sequences of three different species of *Echinorhynchus* (*E. bothniensis* sensu lato, *E. truttae* and *E. gadi*). Furthermore, a single sequence from GenBank of

Table 3. Ranges of measurements conducted on seven female and four male specimens of *E. cinctulus*

|	Male	Female
Trunk		
Length (mm)	3.9 (2.8–4.9)	7.5 (5.5–10.8)
Width	923.5 (850.5–1026)	1.2 (0.9–1.5) (mm)
Proboscis		
Length	702 (544–817.5)	1009 (588–1179)
Width	269 (209–320)	386.3 (346.6–441.8)
Hooks		
Largest hooks	53–56	57–58
Smallest hooks	26–31	28–34
Proboscis receptaculum		
Length	924 (740–1035)	1166 (962–1176)
Width	467.5 (213–840)	321.7 (258.7–361.3)
Lemnisci		
Length	552.5 (482–623)	643 (386.5–828.5)
Width	170 (147.5–192)	231.8 (183.5–318.6)
Testes		
Anterior length	378 (210–497)	
Anterior width	483 (239–773)	
Posterior length	492.5 (404–657)	
Posterior width	345 (241–425)	

Range of measurements are given in parentheses. Measurements are in µm unless otherwise stated.

Echinorhynchus salmonis Müller 1784 falls outside of the main *Echinorhynchus* clade (Fig. 2A).

Concerning *Pomphorhynchus*, results of most samples have been presented earlier (Reier *et al.*, 2019). For the current study, five additional individuals were included, which were assigned to the species *P. bosniacus* and *P. tereticollis*. In the *NJ* tree (Fig. 2A–C) we present all *Pomphorhynchus* sequences from Austria available so far (this study as well as Reier *et al.*, 2019), representing the three species *P. bosniacus*, *P. tereticollis* and *P. laevis*. The sequences of newly analysed individuals cluster in the tree and the *MJ* network with conspecific representatives (46 individuals) detected previously by Reier *et al.* (2019). Both species, *P. bosniacus* and *P. tereticollis*, were found at the same locality in Styria in consecutive years (Table 1). An additional specimen of *P. tereticollis* was obtained from *B. barbatula* from Burgenland. In the haplotype network of *P. tereticollis* the two haplotypes detected in this study are shared with specimens from our previous study from Styria (Fig. 2C). The two specimens belonging to *P. bosniacus* had two different haplotypes, one of which was shared with a specimen from our previous study originating from the river Danube (Vienna) (Fig. 2B). Mean intraspecific genetic *p*-distances are shown in Table 6, compared to Reier *et al.* (2019) they are lower as only sequences of specimens from Austria are included here.

Discussion

In the current study, we confirm the occurrence of six species of fish-parasitizing Acanthocephala in Austria including the first record of *E. cinctulus* in Austria. One species of the genus *Acanthocephalus* does not correspond – according to the DNA
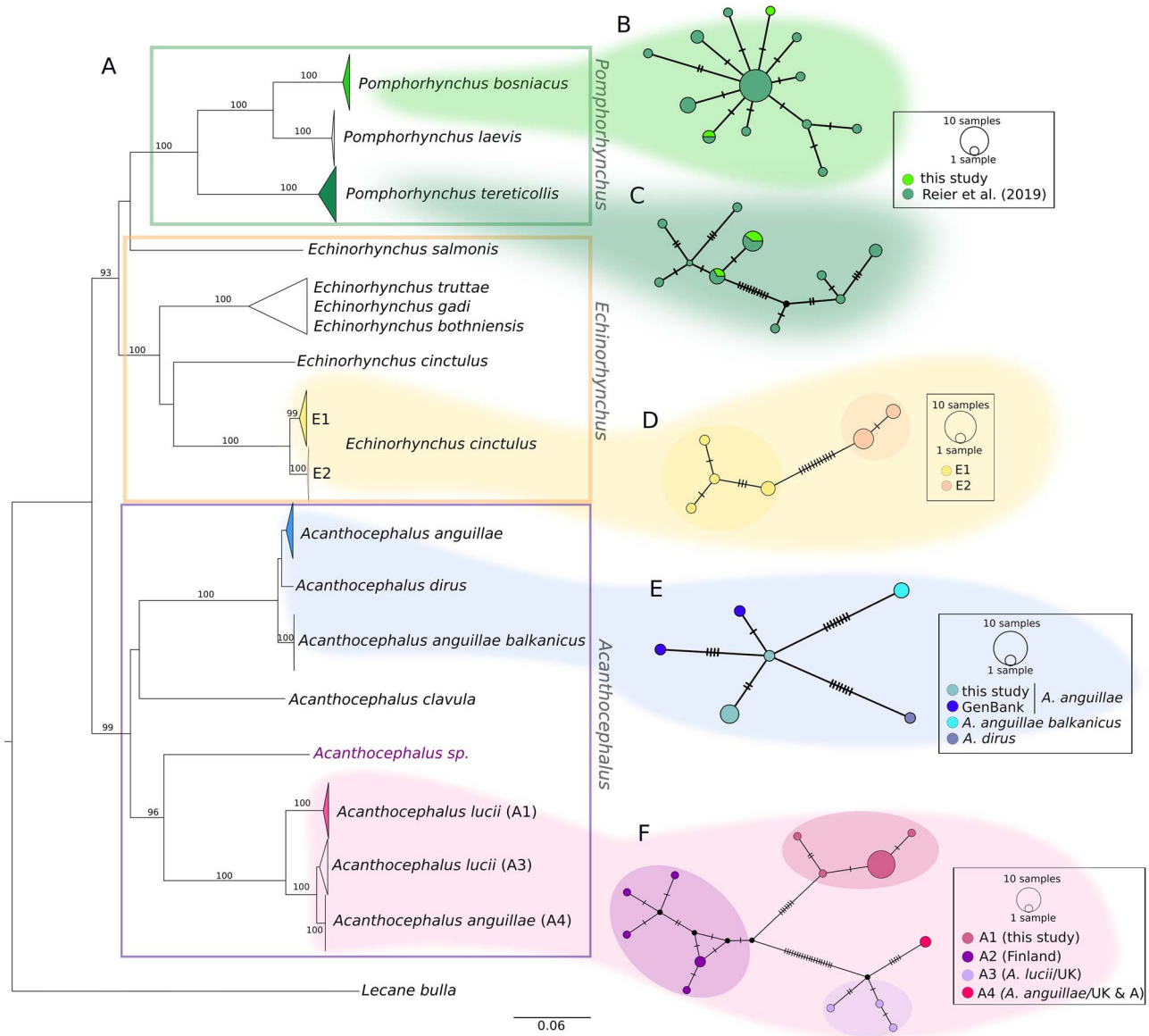
Fig. 2. Phylogenetic relationships between genera of fish-parasitizing Acanthocephala based on a 544 bp *COI* dataset. (A) NJ tree comprising 102 *COI* sequences showing uncorrected *P*-distances between the genera *Pomphorhynchus*, *Echinorhynchus* and *Acanthocephalus*. Bootstrap values [1000 replicates, in %, only values with high support (>80%) are given] are shown next to the nodes. The dataset includes sequences generated in this study and sequences obtained from NCBI GenBank. Clades comprising sequences processed in this study are coloured. (B and C) *MJ* haplotype network of *P. bosniacus* and *P. tereticollis* including sequences of this study and our previous study. (D) *MJ* haplotype network of *E. cinctulus* showing two subclades (E1 and E2) of this study. (E) *MJ* haplotype network of *A. anguillae* sequences from Austria (this study) and Germany (GenBank), *A. anguillae balkanicus* and *A. dirus*. (D) *MJ* haplotype network (428 bp) of *A. lucii* (and two sequences of *A. anguillae*) from different localities. Subclade A1 comprises sequences generated in this study. Mutation steps are indicated with vertical lines. Black dots represent haplotypes missing in the study sampling.

barcode – to any hitherto recorded species from Austria and public databases did not deliver any closely related sequence. Therefore, we could not determine it at the species level. Furthermore, we present the first record of *P. bosniacus* for Styria, a species which was so far only known from the Danube in Austria (Reier *et al.*, 2019).

Occurrences of Acanthocephala in Austria

Three species belonging to the genus *Acanthocephalus* were sampled and identified during this survey. Two of these species, *A. anguillae* and *A. lucii*, were also listed by Kritscher (1985). The third species, of which only one specimen was found, had its proboscis invaginated which made a morphological identification impossible. Since DNA sequence comparisons did not provided any hints, the specimen must remain undetermined and may represent a new species.

Specimens of *A. anguillae* clustered in the phylogenetic tree with *A. dirus* and *A. a. balkanicus* sequences retrieved from GenBank (Fig. 2A). Furthermore, we found a high genetic variation in the haplotypes of *A. anguillae* sensu lato (Fig. 2B). The clustering of *A. dirus* within this clade was unexpected, since it is not yet reported for Europe and known as the most common *Acanthocephalus* species in North America (Amin, 1984, 1985). Also, it has simple hook roots without extensions (e.g. Amin, 1975, 1984; Golvan, 1969), while *A. anguillae* possess two lateral extensions as observed in the specimens of this study. Therefore, the assignment of the specimens determined in this study as *A. anguillae* was straightforward. The clustering of *A. dirus* within the *A. anguillae* clade was already attributed to a probable misidentification by Amin *et al.* (2019). The isopod *Asellus aquaticus* (L.) was reported as the host of the latter specimen (García-Varela and Nadler, 2006) and, since the life cycle of these helminths is not completed yet in the intermediate host, the specimen

Table 4. Genetic distances (*p*-distances in %) for the *COI* dataset of the genus *Acanthocephalus*

Species	No. of sequences	Ø distances within groups	Max. distances	A. anguillae	Acanthocephalus sp.	A. lucii (A1)
A. anguillae	6	0.5	0.4			
Acanthocephalus sp.	1			27.8 (27.2–27.9)		24.1 (24.1–24.4)
A. lucii (A1)	16	0.1	0.7	29.12 (29.2–29.7)	24.2 (24.1–24.4)	
A. dirus	1			1.6 (1.3–1.7)	27.4	29.3 (29.2–29.6)
A. anguillae balkanicus	2	0	–	1.8 (1.6–2.2)	28.5–28.6	29.4
A. clavula	1			(23.7–23.8)	24.6	27.3 (27.2–27.6)
A. lucii (A3)	2	0.6	0.9	27.9 (27.6–28.3)	24 (23.7–24.6)	6.3 (6.2–6.4)
A. anguillae (A4)	3	0	–	28.3 (27.7–28.5)	24.6	6.2 (6.1–6.4)
A. lucii (A2)*	6	1	1.1			2.4 (2.1–2.6)

Groups containing specimens of this study are highlighted in grey. Sequences of *A. lucii* (A2) derived from GenBank based on a shorter alignment are indicated with an asterisk.

probably was at larval stage which might have impeded correct morphological identification. Another reason for misidentification might be due to morphological variation within *A. anguillae* sensu lato: the two lateral extensions were previously described as one of the most characteristic identification traits of the species (e.g. Meyer, 1933; Petrochenko, 1956), but they were not observed (or only rudimentary) in *A. a. balkanicus*, a recently described subspecies of *A. anguillae* parasitizing in *Proteus anguinus* Laurenti, 1768 (Amin et al., 2019). Therefore, the specimen from the USA could represent an additional lineage of *A. anguillae* sensu lato which lacks the lateral extensions, too. Also, the occurrence of *A. anguillae* in North America might be possible (Meyer, 1933; Petrochenko, 1956).

The common species *A. lucii* was collected in Lower Austria and in Burgenland. The shape of the hook roots as well as the anatomy corresponds to previous descriptions of *A. lucii* in the literature (Meyer, 1933; Petrochenko, 1956; Golvan, 1969). Sequences of the *COI* marker of *A. lucii* from Finland (approximately 450 bp; Benesh et al., 2006) were included in the *MJ* network analysis. They form a separate subclade (A2) closely related to subclade A1 (*P*-distance 2.5%), which supports their taxonomic assignment. However, *NJ* analysis revealed a considerably high genetic *p*-distance of 6.2% between our specimens and sequences derived from GenBank determined as *A. lucii* from the UK (subclade A3). Benesh et al. (2006) mentioned the option of a misidentification of the English specimens or the existence of an English strain of *A. lucii*. The latter assumption is supported by an English sequence of another study determined as *A. lucii* (Wayland et al., 2015), found in the same subclade (A3).

Interestingly, two GenBank sequences of *A. anguillae* from the UK and Austria (subclade A4) occurred within the *A. lucii* clade what had been explained by mitochondrial (*mt*) introgression due to the co-existence of the two species in one host (Benesh et al., 2006). Competitive behaviour between the two species has been reported, whereby *A. lucii* seemed to be the stronger competitor (Kennedy, 1992). In Austria, a co-existence of *A. anguillae* and *A. lucii* in European Eels was observed in Lake Neusiedl in Illmitz (Burgenland) during a long-term survey (Schabuss et al., 2005). Results of the latter study did not indicate an overrepresentation of *A. lucii* in relation to *A. anguillae*, but an alternating presence of the two species in Illmitz with a higher abundance of *A. anguillae* in spring and a switch in autumn, when *A. lucii* becomes the dominant species (Schabuss et al., 2005). Anyhow, the high genetic distance between *A. lucii* and *A. anguillae* (mean *p*-distance of 29.3%) argues against an *mt* introgression between the two species. Therefore, it is more likely that the

sequences of clade A4 were misidentified as *A. anguillae*. Also, the origin of the sequence available in GenBank, determined as *A. anguillae* from Austria would be interesting. As there is no information available on morphology of the published sequences in clade A3 and A4, their taxonomic assignment remains open.

Benesh et al. (2006) mentioned the problem of nuclear pseudogenes (numts) of the mitochondrial *COI* gene in *A. lucii*. However, in the current study indications for numts were not encountered. The use of nested primers specific for *A. lucii* might have overcome such problems.

We found one species of *Echinorhynchus* in the current study. Of the two subclades of *E. cinctulus*, one occurred in the river Mur in Styria (E1) and the other one in Lower Austria in brooks around Lunz entering the river Ybbs (E2). The genetic *p*-distance of 2.9% between the two subclades E1 and E2 implies the existence of a phylogeographic structure. Considerable intraspecific variation was observed in some Acanthocephala and were especially investigated in the genus *Pomphorhynchus* (Dudiňák and Šnábel, 2001; O'Mahony et al., 2004; Vardić Smrzlić et al., 2015; Perrot-Minnot et al., 2018) as well as in *Polymorphus minutus* sensu lato (Zittel et al., 2018; Grabner et al., 2020). Also, intraspecific differences were revealed for species of *Echinorhynchus*, explained by post-glacial population bottlenecks (Väinölä et al., 1994). Unfortunately, due to the juvenile life stage of individuals of subclade E1, a more detailed morphological comparison of the two lineages was impossible. The examined specimens of lineage E2 were adult and their thorough morphological investigation indicated that the measurements were in concordance with the detailed description of *E. cinctulus* (formerly *E. borealis*; Grabda-Kazubska and Ejsymont, 1969). *Echinorhynchus borealis* was synonymized with *E. cinctulus* since the name *E. borealis* was preoccupied by *Echinorhynchus borealis* Gmelin 1791 (Golvan, 1994; Amin, 2013). The high genetic distance to a *COI* sequence from GenBank assigned to *E. cinctulus* (20%) raised doubts on whether these two distinct mitochondrial lineages belong to the same species. Cryptic species are known for various species of Acanthocephala (e.g. Steinauer et al., 2007; Zittel et al., 2018). For example, the wide distances found between mitochondrial lineages of *P. laevis* (up to 20%) lead to the assumption of cryptic species (Perrot-Minnot et al., 2018; Reier et al., 2019). Remarkable morphological variation within *E. cinctulus* was revealed in the work of Grabda-Kazubska and Ejsymont (1969) and it presently remains unknown whether the authors examined cryptic species or if the species is featured by a high intraspecific variation. The specimen of *E. cinctulus* processed by Wayland et al. (2015) was obtained from *L. lota* in Finland. Wayland

Table 5. Genetic distances (*P*-distances in %) for the *COI* dataset of the genus *Echinorhynchus*

Species	No. of sequences	Ø distances within groups	Max. distances	*E. cinctulus* (E1)	*E. cinctulus* (E2)
E. cinctulus (E1)	5	0.5	0.7		
E. cinctulus (E2)	6	0.1	0.2	2.2–2.9	
E. cinctulus (GenBank)	1			19.8–20.4	19.6–19.8
E. salmonis	1			27.2. – 27.5	27–27.2
Echinorhynchus spp.	5	6.4	7.9	20.9–22.9	21.3–22.9

Groups containing specimens of this study are highlighted in grey.

et al. (2015) considered the systematics of the whole genus *Echinorhynchus* as controversial and recommended a total revision of *Echinorhynchus* sensu lato (Petrochenko, 1956; Golvan, 1969) by using an integrative taxonomic approach and to split the genus into smaller units, if monophyletic groups with distinct morphological traits can be distinguished. Our results confirm the need for a comprehensive survey of *Echinorhynchus* spp. throughout Europe gathering enough material to assess geographic variation regarding morphology, genetics as well as host range. Until now, *E. truttae* has been described as only representative of *Echinorhynchus* s. l. in Austria (Kritscher, 1985). The taxonomic status of the specimens determined by Prof O. N. Bauer as *E. borealis*, as reported by Rydlo (1990) remains unconfirmed. The synonymy of *E. borealis* and *E. cinctulus* (Golvan, 1994; Amin, 2013) indicates that the specimens determined by Bauer also belong to *E. cinctulus*. According to all available data, cryptic diversity within and taxonomic status of *E. cinctulus* remains open.

The identified specimens of *Pomphorhynchus* clustered in the known clades from previous studies (e.g. David *et al.*, 2017; Perrot-Minnot *et al.*, 2018; Reier *et al.*, 2019). The two species *P. tereticollis* and *P. bosniacus* were distinguished. Both species were found in a fish farm in consecutive years in rainbow trouts: *P. bosniacus* was obtained during 2017, whereas *P. tereticollis* was found during the year 2018. The question of whether both species co-exist in the fish farm or whether one species replaced the other in the following year could not be clarified. An explanation for the alternating occurrence of the two parasite species could be the purchase and stocking of fish from different suppliers during shortages (fish farmer, personal communication). Another scenario might be the introduction of cystacanths through intermediate hosts, since the fish farm is connected to white water. Interestingly, the specimens belonging to *P. tereticollis* shared haplotypes with specimens obtained from fish caught in the nearby Styrian rivers Mur and Sulm in former years (Reier *et al.*, 2019; Fig. 2C). This could be an indication that the specimens in the fish farm derived from white water. A replacement of dominant species was also reported in other studies (Schabuss *et al.*, 2005). The specimens belonging to *P. bosniacus* were closely related to specimens from the Danube obtained in former years (Fig. 2B), which could be an indication that they were introduced into the fish farm. Nevertheless, it is important to note that one very common haplotype is shared by many specimens of *P. bosniacus* sampled along the Danube and, moreover, many closely related haplotypes (one to two mutation steps to this common haplotype) were detected in various European countries (Reier *et al.*, 2019). Therefore, it is difficult to trace the origins of haplotypes of this species. A long-term study in correlation with data of fish stocking and a monitoring of the potential intermediate host fauna should be conducted to elucidate the alternating occurrences in fish farming.

Pomphorhynchus tereticollis was the most frequently detected species of *Pomphorhynchus* in Styria in a previous study (Reier

et al., 2019). *Pomphorhynchus laevis* which was previously detected in Styria (Reier *et al.*, 2019) was not recorded in the current study. For *P. bosniacus* we report here the first record for Styria. Furthermore, we report the occurrence of *P. tereticollis* in Burgenland with *B. barbatula* as host species.

Reliability of DNA barcodes

Identification of helminths based on morphology is challenging, and the taxonomic expertise is mostly restricted to a few persons. Not without reason Acanthocephala are denoted as 'a taxonomists nightmare' (Kennedy, 2006), showing high individual variability in proboscis size, and number and arrangement of the hooks on the proboscis (Cleave, 1952), which are respectively the only hard structures on their body (e.g. Brown, 1987; Kennedy, 2006). Reliable DNA barcodes can simplify species assignment of e.g. animals with invaginated proboscis or damaged specimens or of larval stages (Alcántar-Escalera *et al.*, 2013). Meyer and Paulay (2005) criticized the solely usefulness of DNA barcoding in well-examined and efficient sampled taxa, which was supported in this study for the phylum Acanthocephala. It is a known issue that there exists a high number of sequences with wrong taxonomic determination in GenBank since quality checks are not standard and it is not mandatory to provide details on the origin of the specimen from which a sequence was obtained (Harris, 2003). The BOLD data systems established more strict requirements (Ratnasingham and Hebert, 2007), but since data are also mined from GenBank, sequences of misidentified specimens accumulate there, too. This, in turn, could lead scientists to draw false conclusions from published sequences.

Indeed, our study indicated that DNA barcoding might be a promising tool in the identification of Acanthocephala, as shown for the genus *Pomphoryhnchus*. Yet, only a thorough morphological investigation followed by the determination of DNA barcodes (Reier *et al.*, 2019) provided reliable basis for subsequent species identification. Inconsistencies and uncertain species assignment as shown for the genera *Acanthocephalus* and *Echinorhynchus* underline the need for more DNA sequence data of morphologically investigated and documented specimens. Therefore, DNA sequence data should be used with caution when no morphological data are provided.

Conclusion

Our study showed that DNA barcoding might be a promising tool in the identification of parasitic taxa, but for the taxon Acanthocephala we are still far from being able to use DNA barcoding for species identification. There still exist species with an uncertain species validity (Amin, 2013) for which it is recommended to use a combined approach by investigating freshly collected material as well as museum collection material as references. Furthermore, our study further indicates cryptic species within taxa of Acanthocephala. Therefore, we suggest a

Table 6. Genetic distances (*p*-distances in %) for the *COI* dataset of the genus *Pomphorhynchus*

Species	No. of sequences	Ø distances within groups	Max. distances	P. tereticollis	P. bosniacus
P. tereticollis	15	1.2	2.2		
P. bosniacus	24	0.2	0.3	22.7 (22.2–23.3)	
P. laevis	10	0.1	0.2	20.1 (19.5–20.4)	10.4 (10.1–10.8)

Groups containing specimens of this study are highlighted in grey.

revision of the phylum in Europe, based on an integrative taxonomic approach, and a broad sampling throughout Europe should be conducted to establish reliable DNA barcodes for Acanthocephala.

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