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

Susanne Reier1,2, Helmut Sattmann3, Thomas Schwaha2, Hans-Peter Fuehrer4 and Elisabeth Haring1,2

1Central Research Laboratories, Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria; 2Department of Evolutionary Biology, University of Vienna, Althanstr. 14, 1090 Vienna, Austria; 33rd Zoological Department, Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria and 4Department of Pathobiology, Institute of Parasitology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

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 Koechleurer, 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; Reiter 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 Kritschker (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 truncatus Schrank, 1788 and Pomphorhynchus laevis (Müller, 1776), all belonging to the class Palaeacanthocephala (Kritschker, 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 bosnicus 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 were 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. bosnicus (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, lemmisci 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 QiAamp 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 (Girbet et al., 2004), DQ089710 (García-Varela and Nadler, 2006)]. The rotifer Lecane bulbula (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. bosnicus, E. cinctulus, A. anguilae 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
| Lab          | Museum | BOLD       | GenBank     | Host species | Waterbody                  |
|--------------|--------|------------|-------------|--------------|----------------------------|
| 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           |
| 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   |

**Pomphorhynchus bosniacus**

| Lab          | Museum | BOLD       | GenBank     | Host species | Waterbody                  |
|--------------|--------|------------|-------------|--------------|----------------------------|
| BF-P-003     | 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           |

**Echinorhynchus cinctulus**

| Lab          | Museum | BOLD       | GenBank     | Host species | Waterbody                  |
|--------------|--------|------------|-------------|--------------|----------------------------|
| 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 anguillae**

| Lab          | Museum | BOLD       | GenBank     | Host species | Waterbody                  |
|--------------|--------|------------|-------------|--------------|----------------------------|
| BA2-3        | 21297/1| ACANT066-20| MT682936    | Perca fluviatilis | Waidhofen/T. (fish pond), LA |
| BA1          | 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.
from Finland [AM039878, AM039880], 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. luci* and *A. anguillae* according to characters described in the literature (Meyer, 1933; Petrochenko, 1956; Golvan, 1969). Four specimens of *A. anguillae* [from Anguilla anguillae (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 Anguilla, Gymnocephalus cernua (L.) and Perca fluviatilis (L.)] were determined as *A. luci*. Morphological determination was straightforward since the analyzed 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. luci*. 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. anguillla*) 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).

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. luci*) | | | | |
| H1AcanCOIFw1 | TTCTACAAATCTAAARGATATGGG | 48 | ≤540 | Reier et al. (2019) |
| H1AcanCOIRv2 | AAAAAATAAMACTCCAGGATGACAAAA | | | Reier et al. (2019) |
| COI amplification *A. luci* | | | | |
| Acan-3′ | TTAGTGCCGTATCATTGAG | 56 | ≤540 | This study |
| Acan-4′ | ACAAGACTTTTGGACCATGCAGGA | | | This study |
| Sequencing primers *P. tereticollis* | | | | |
| P_tere_COI_fw | TTATGGGGTTTTCTATAAGG | 50 | ≤650 | This study |
| H1AcanCOIRv2 | AAAATAAMACTCCAGGATGACAAAA | | | Reier et al. (2019) |
| Sequencing primers *P. bosniacus* | | | | |
| P_bos_COI_fw | CTAATGGGGTTTTCTATAAGG | 50 | ≤650 | This study |
| H1AcanCOIRv2 | AAAATAAMACTCCAGGATGACAAAA | | | Reier et al. (2019) |

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-Kazub ska 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 Onchorhynchus 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 Acanthocephalus 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
null
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 provide 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

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 *A. anguillae* sequence 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.
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. balkanica*, 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. anguillae* 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* (Dudíňák and Šnábel, 2001; O’Mahony et al., 2004; Vardić Smrzlić et al., 2015; Perrot-Minnit 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-Minnit 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

| Species                  | No. of sequences | Ø distances within groups | Max. distances | A. anguillae | Acanthocephalus sp. | A. lucii (A1) |
|--------------------------|------------------|---------------------------|----------------|--------------|---------------------|---------------|
| *A. anguillae*           | 6                | 0.5                       | 0.4            | 27.8 (27.2–27.9) | 24.1 (24.1–24.4)   |               |
| Acanthocephalus sp.      |                  |                           |                |              |                     |               |
| *A. lucii* (A1)          | 16               | 0.1                       | 0.7            | 29.12 (29.2–29.7) | 24.2 (24.1–24.4)   |               |
| *A. dirus*               | 1                | 1                         | 1.6            | 1.3–1.7      | 27.4                | 29.3 (29.2–29.6) |
| *A. anguillae* balkanica |                  |                           |                |              |                     |               |
| *A. clavula*             | 1                | 0                         |                | 1.8 (1.6–2.2)  | 28.5–28.6           | 29.4          |
| *A. lucii* (A3)          | 2                | 0.6                       | 0.9            | 27.3 (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            | 2.6          | 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.
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 Pomphorhynchus. 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 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              |                  |                            | 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.
revison 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.

**Supplementary material.** The supplementary material for this article can be found at [https://doi.org/10.1017/S0033182020001316](https://doi.org/10.1017/S0033182020001316)

**Acknowledgements.** We thank Julia Schindler and Barbara Tautscher for technical assistance in the lab. We also express our gratitude to Stefan Koblmiiller and Lukas Zangl of the University of Graz, Robert Koneczy of the Environment Agency Austria, Michael Schabuss of Profisch OG, Vienna, Nadine Ehm of WasserCluster Lunz and Clemens Gumpinger of blatt-fisch e.U., Wels, for providing material and valuable discussions.

**Financial support.** Financial support was provided by the Austrian Federal Ministry of Education, Science and Research via the ABOL initiative (Austrian Barcode of Life; www.abol.ac.at). SR was supported by the FEMtech initiative of the Austrian Federal Ministry for Transport, Innovation and Technology (BMVIT). Open access funding provided by the University of Vienna.

**Conflict of interest.** None.

**Ethical standards.** None.

**References**

Alcantar-Escalera FJ, García-Varela M, Vázquez-Domínguez E and Pérez-Ponce de León G (2013) Using DNA barcoding to link cyctanths and adults of the acanthocephalan Polymorphus brevis in central Mexico. *Molecular Ecology Resources* 13, 1116–1124.  
Amin OM (1975) Acanthocephalus parksidei sp. n. (Acanthocephala: Echinorhynchidae) from Wisconsin Fishes. *The Journal of Parasitology* 61, 301–306.  
Amin OM (1984) Variability and redescription of *Acanthocephalus dirus* (Acanthocephala: Echinorhynchidae) from freshwater fishes in North America. *Proceedings of the Helminthological Society of Washington* 51, 225–237.  
Amin OM (1985) Hosts and geographic distribution of *Acanthocephalus* (Acanthocephala: Echinorhynchidae) from North American freshwater fishes, with a discussion of species relationships. *Proceedings of the Helminthological Society of Washington* 52, 210–220.  
Amin OM (2013) Classification of the Acanthocephala. *Folia Parasitologica* 60, 273–305.  
Amin OM, Heckmann RA, Fiser Z, Zaksek V, Herlyn H and Kostanjsek R (2019) Description of *Acanthocephalus anguillae* balkanicus subsp. n. (Acanthocephala: Echinorhynchidae) from *Proteus anguinus* Laurenti (Amphibia: Proteidae) and the cave ecomorph of *Asellus aquaticus* (Crustacea: Asellidae) in Slovenia. *Folia Parasitologica* 66, 015.  
Bandelt HJ, Forster P and Rohl A (1999) Median-joining networks for inferring intraspecifc phylogenies. *Molecular Biology and Evolution* 16, 37–48.  
Benesh DP, Hasu T, Suomalainen L-R, Valtonen ET and Tiirula M (2006) Reliability of mitochondrial DNA in an acanthocephalan: the problem of pseudogenes. *International Journal for Parasitology* 36, 247–254.  
Brown AF (1987) Anatomical variability and secondary sexual characteristics in *Pomphorhynchus laevis* (Müller, 1776) (Acanthocephala). *Systematic Parasitology* 9, 213–219.  
Cleave HIV (1952) Speciation and formation of genera in acanthocephalans. *Systematic Zoology* 1, 72.  
David GM, Staettzel C, Schlumberger O, Perrot-Minnot M-J, Beisel J-N and Hardion L (2017) A minimalistic macroparasite diversity in the round goby of the Upper Rhine reduced to an exotic acanthocephalan lineage. *Parasitology* 145, 1–7.  
Dudínák V and Snábel V (2001) Comparative analysis of Slovak and Czech populations of *Pomphorhynchus laevis* (Acanthocephala) using morphological and isoenzyme analyses. *Acta Zoologica Universitatis Comenianae* 44, 41–50.  
García-Varela M and Nadler SA (2006) Phylogenetic relationships among Syndermata inferred from nuclear and mitochondrial gene sequences. *Molecular Phylogenetics and Evolution* 40, 61–72.  
Gibson D, Bray R, Hunt D, Georgiev B, Scholz T, Harris P, Bakke T, Pojmanska T, Niewiadomska K, Kostadinova A, Tkach V, Bain O, Durette-Desset M-C, Gibbons L, Moravec F, Petter A, Dimitrova Z, Buchmann K, Valtonen E and de Jong Y (2014) Fauna Europea: helminths (animal parasitic). *Biodiversity Data Journal* 2, e1060.  
Giribet G, Sorensen MV, Funch P, Kristensen RM and Sterrer W (2004) Investigations into the phylogenetic position of Micrognathozoa using four molecular loci. *Cladistics* 20, 1–13.  
Golvan VJ (1969) *Systematique des acanthocephales* (Acanthocephala Rudolphi 1801). Première partie: l’ordre des Palaeacanthocephala Meyer 1931, premier fascicule: la super-famille des Echinorhynchoidea (Cobbold 1876) *Golvan Et Mouin 1963. Memoires. Museum National d’Histoire Naturelle, Serie A, Zoologie* 57, 1–373.  
Golvan VJ (1994) Nomenclature of the Acanthocephala. *Research and Reviews in Parasitology* 34, 135–205.  
Grabda-Kazubska B and Ejsymont L (1989) Studies on morphology, variability and systematic status of *Echinorhynchus borealis* Linstow, 1901 (Acanthocephala, Echinorhynchidae). *Acta Parasitologica Polonica* 17, 65–87.  
Grabner D, Doliwa A, Bulantová J, Horák P and Sures B (2020) Morphological comparison of genetically differentiated *Polymorphus* Cf. minutus types. *Parasitology Research* 119, 153–163.  
Harris D (2003) Can you bank on GenBank? *Trends in Ecology & Evolution* 18, 317–319.  
Hohenadler MAA, Nachev M, Thielen F, Taraschewski H, Grabner D and Sures B (2017) *Pomphorhynchus laevis*: an invasive species in the river Rhine? *Biological Invasions* 20, 207–217.  
Kennedy CR (1992) Field evidence for interactions between the acanthocephals *Acanthocephalus anguillae* and *A. lucii* in eels, *Anguilla anguilla*. *Ecological Parasitology* 1, 122–134.  
Kennedy CR (2006) *Ecology of the Acanthocephala*. Cambridge, UK: Cambridge University Press, 249 pp.  
Kritscher E (1985) Teil 4, Aschelminthes, Schlauchwürmer: Nemertini, Schnuwwérer: d. Phylum: Acanthocephala. In: Österreichische Akademie der Wissenschaften: *Catalogus Faunae Austriae*: Ein Systematisches Verzeichnis Aller auf Österreichischem Gebiet Festgestellten Tierarten. Wien, Verlag der Österreichischen Akademie der Wissenschaften, 15 pp.  
Kumar S, Stecher G, Li M, Knyaz C and Tamura K (2018) *MEGA* X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35, 1547–1549.  
Ließe M (1911). Heft 16: Acanthocephalen. Register der Acanthocephalen Ordnungen des Tier-Reichs, Band 4, Abt. 2, Lief. 1. Leipzig, Akademische Verlagsgesellschaft: 1–322 pp.; Ließ 2, Leipzig, Akademische Verlagsgesellschaft: 333–582 pp.  
Meyer CP and Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology* 3, e422.  
O’Mahony EM, Bradley DG, Kennedy CR and Holland CV (2004) Evidence for the hypothesis of strain formation in *Pomphorhynchus laevis*
(Acanthocephala): an investigation using mitochondrial DNA sequences. Parasitology 129, 341–347.

Perrot-Minnot M-J, Špakulová M, Wattier R, Kotlík P, Dişen S, Aydoğdu A and Tougard C (2018) Contrasting phylogeography of two Western Palaearctic fish parasites despite similar life cycles. Journal of Biogeography 45, 101–115.

Petroschenko VI (1956) Acanthocephala of domestic and wild animals. Vol. I. Moscow: Izdatelstvo Akademii Nauk SSR (English translation by Israel Program of Scientific Translations, Keter Press, Jerusalem, Israel, 1971). 465 pp.

Ratnasingham S and Hebert PD (2007) BOLD: the barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7, 355–364.

Reier S, Sattmann H, Schwaha T, Harl J, Konecny R and Haring E (2020) An integrative taxonomic approach to reveal the status of the genus Pomphorhynchus Monticelli, 1905 (Acanthocephala: Pomphorhynchidae) in Austria. International Journal for Parasitology: Parasites and Wildlife 8, 145–155.

Reier S, Sattmann H and Haring E (2020) Optimizing conflicting tasks in the analysis of parasitic worms: morphological imaging, DNA yield, specimen and DNA preservation. Annalen des Naturhistorischen Museums in Wien. Serie B für Botanik und Zoologie 94, 41–45.

Schabuss M, Kennedy CR, Konecny R, Grillitsch B, Reckendorfer W, Schiemer F and Herzig A (2005) Dynamics and predicted decline of Anguillicola crassus infection in European eels, Anguilla anguilla, in Neusiedler see, Austria. Journal of Helminthology 79, 159–167.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P and Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682.

Špakulová M, Perrot-Minnot M and Neuhaus B (2011) Resurrection of Pomphorhynchus tereticollis (Rudolphi, 1809) (Acanthocephala: Pomphorhynchidae) based on new morphological and molecular data. Helminthologia 48, 268–277.

Steinauer ML, Nickol BB and Ortí G (2007) Cryptic speciation and patterns of phenotypic variation of a highly variable acanthocephalan parasite: patterns of phenotypic variation. Molecular Ecology 16, 4097–4109.

Väinölä R, Valtonen ET and Gibson DI (1994) Molecular systematics in the acanthocephalan genus Echinorhynchus (Sensu lato) in Northern Europe. Parasitology 108, 105–114.

Vardić Smrzlić I, Valić D, Kapetanović D, Filipović Marijić V, Gjurićević E and Teskeredžić E (2015) Pomphorhynchus laevis (Acanthocephala) from the Sava River basin: new insights into strain formation, mtDNA-like sequences and dynamics of infection. Parasitology International 64, 243–250.

Wayland MT, Vainio JK, Gibson DI, Herniou EA, Littlewood DTJ and Väinölä R (2015) The systematics of Echinorhynchus Zoega in Müller, 1776 (Acanthocephala, Echinorhynchidae) elucidated by nuclear and mitochondrial sequence data from eight European taxa. ZooKeys 484, 25–52.

Zittel M, Grabner D, Wleklik A, Sures B, Leese F, Taraschewski H and Weigand AM (2018) Cryptic species and their utilization of indigenous and non-indigenous intermediate hosts in the acanthocephalan Polymorphus minutus sensu lato (Polymorphidae). Parasitology 145, 1421–1429.