Research Article

Cryptic species and their utilization of indigenous and non-indigenous intermediate hosts in the acanthocephalan *Polymorphus minutus sensu lato* (Polymorphidae)

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Abstract

The bird-infecting acanthocephalan *Polymorphus minutus* has been suggested to comprise different lineages or even cryptic species using different intermediate hosts. To clarify this open question, we investigated *Polymorphus* cf. *minutus* cystacanths originating from amphipod intermediate hosts from 27 sites in Germany and France. Parasites and hosts were identified using integrated datasets (COI and/or morphology for hosts and COI + ITS1-5.8S-ITS2 for parasites).

Mitochondrial and nuclear data (ITS1) strongly support the existence of three cryptic species in *Polymorphus* cf. *minutus* (type 1-3). These three types reveal a high degree of intermediate host specificity, with *Polymorphus* type 1 only encountered in *Gammarus fossarum* type B, *Polymorphus* type 2 in *Echinogammarus* sp. and *Echinogammarus berilloni*, and *Polymorphus* type 3 in *Gammarus pulex* and *Gammarus roeselii*. Our results point to a so far neglected cryptic diversity of the genus *Polymorphus* in Central Europe. Furthermore, *Polymorphus* type 2 is most likely a non-native parasite in Germany that co-invaded with *E. berilloni* from the Mediterranean area. Potentially, type 3 originates from South-East Europe and migrated to Germany by *G. roeselii*, where it might have captured *G. pulex* as an intermediate host. Therefore, our findings can be seen in the context of ecological globalization in terms of the anthropogenic displacement of intermediate hosts and its impact on the genetic divergence of the parasites.

Introduction

In the last years, cryptic parasite species were continuously discovered in various helminth groups (Pérez-Ponce de León and Poulin, 2017). This can mainly be attributed to the application of molecular techniques that often provide a higher taxonomic resolution than morphological characters. The same trend was also found within the Acanthocephala, with several cryptic species discovered in various genera (e.g. Steinauer *et al.* 2007; Wayland *et al.* 2015). Acanthocephalans have a complex life cycle, including at least one obligatory host change. Arthropods, such as crustaceans or insects, serve as intermediate hosts whereas fish, birds, mammals and amphibians are known as final hosts, where reproduction occurs in the intestine. In some species, additional paratenic hosts can exist (Taraschewski, 2000; Kennedy, 2006; Sures, 2014). Acanthocephalans belonging to the genus *Polymorphus* have an aquatic life cycle with waterfowl as final hosts in most species. In their amphipod intermediate hosts, the parasites migrate into the haemocoel where they develop to the cystacanth stage. The final host gets infected by ingesting the infected amphipod (Taraschewski, 2000; Kennedy, 2006). The life cycles of some species of the Polymorphidae have not been fully understood to date and molecular barcoding has been applied successfully to link parasite stages in the intermediate and definitive hosts (Alcántar-Escalera *et al.* 2013).

The species *Polymorphus minutus* was originally described in Germany by Goeze (1782) and later found in a wide variety of aquatic birds (e.g. Lühe, 1911). Since the elucidation of the complete life cycle of *P. minutus*, various species of freshwater amphipods were identified as intermediate hosts (Greef, 1864; Schmidt, 1985). An experimental transmission study with *P. minutus* from the 1950s indicated that there is a possible differentiation in lineages or even cryptic species displaying different intermediate host specificities (Hynes and Nicholas, 1958): *P. minutus* cystacanths isolated from *Gammarus duebeni*, *Gammarus pulex* and *Gammarus lacustris* were used to infect domestic ducks, respectively. The resulting eggs obtained from the adult parasites were used to infect each of the three *Gammarus* spp. The results clearly showed that the eggs were most infective for the same *Gammarus* species from which the respective cystacanths originated. Despite these observations, it was commonly assumed that

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specimens of *Polymorphus* collected in Central Europe all belong to a single species. Accordingly, no recent taxonomic studies were published for this species. Furthermore, *P. minutus* has been frequently used as a model organism in various fields throughout the last decades, including studies about tegumental structure of adults (Crompton and Lee, 1965) and acanthor-larvae (Albrecht *et al.* 1997) or the relation of the parasite with its intermediate and final hosts (Hynes and Nicholas, 1963; Itämies *et al.* 1980; Bollache *et al.* 2001; Bauer *et al.* 2005; Haine *et al.* 2005; Médoc *et al.* 2006; Tain *et al.* 2006; Jacquin *et al.* 2014). If the hypothesis holds true that several cryptic species are subsumed in a presumed 'P. minutus-complex', this will have consequences for the conclusions drawn from behavioural or structural studies, especially if different intermediate host species were involved. Therefore, it is particularly essential to resolve the taxonomy of *Polymorphus* cf. *minutus*, also bearing in mind that this species can lead to high mortalities in waterfowl populations and is recognized as an economically significant pest in goose and duck farming (Hynes and Nicholas, 1963; Itämies *et al.* 1980). However, the morphological differentiation of *Polymorphus* species is difficult, especially based on the cystacanth stage (Alcántar-Escalera *et al.* 2013). Furthermore, closely related parasite species might not provide defined and easily visible morphological characters for an unambiguous species diagnosis (Nadler and Pérez-Ponce de Léon, 2011; Selbach *et al.* 2015). Therefore, we used molecular data – mitochondrial cytochrome c oxidase subunit 1 (COI) fragment and the nuclear internal transcribed spacer (ITS)1-5.8S-ITS2 region – to identify and distinguish the *Polymorphus*-isolates sampled from amphipod populations in Germany and France. The aim was to assess the genetic diversity of what is considered *P. minutus* and its linkage to different intermediate amphipod

hosts, thereby testing previous results from transmission experiments which pointed to a so far neglected cryptic species diversity (Hynes and Nicholas, 1958).

Materials and methods

Amphipod and parasite sampling

Gammarids infected with acanthocephalan cystacanths were sampled by kick-sampling, using a net with a mesh size of 0.3 mm, between 2014 and 2016 at 27 sites in North-Rhine Westphalia, Germany (smaller tributaries of Lippe, Emscher, Ruhr and Rhine), Baden-Wuerttemberg and Rhineland-Palatinate, Germany (tributaries of the Rhine), as well as Île-de-France, France (Loing, tributary of Seine River) (see map in Fig. 1). A list of all sites, sampling dates and the number of sequenced host individuals and *Polymorphus* cystacanths, as well as the geographic location, is given in Supplementary Table S1.

Infected individuals were sorted at the site according to the obvious red coloration of the cystacanths and stored in 95% ethanol. In the laboratory, amphipods were identified morphologically (i.e. for. *Gammarus roeselii* and *Echinogammarus* sp.) according to the taxonomic keys of Eggers and Martens (2001, 2004). Due to morphological difficulties to identify species of the *G. pulex* and *G. fossarum* cryptic species complexes, those were DNA barcoded. The material of the host was not retained in all cases (e.g. when additional collection material was included which had been collected before the study was designed), therefore, a genetic identification of hosts was not always possible (see Supplementary Table S1).
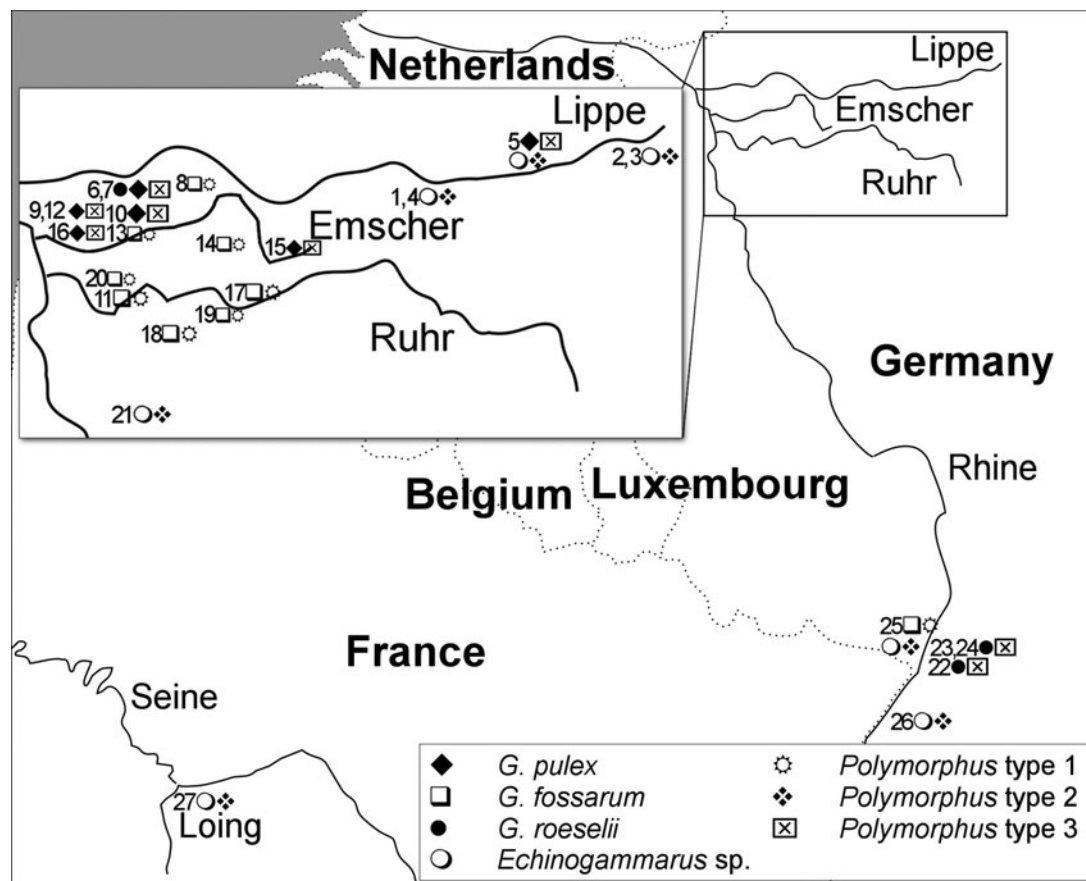

Fig. 1. Map of sampling area. The map shows the major river catchments, the approximate location of the sampling sites with the amphipod and *Polymorphus* types found. Sites 1–8: Lippe (Rhine), 9–16: Emscher (Rhine), 17–20: Ruhr (Rhine), 21–26: small tributaries of the Rhine, 27: Loing (Seine).

PCR and sequencing

Cystacanths were dissected from gammarids and DNA extraction of hosts was performed according to Grabner *et al.* (2015). DNA from small pieces of the cystacanths was extracted with the same protocol. The standard animal barcoding locus COI was amplified for amphipods and acanthocephalans using the degenerated primer pair LCO1490-JJ (5′-CHA CWA AYC ATA AAG ATA TYG G-3′) and HCO2198-JJ (5′-AWA CTT CVG GRT GVC CAA ARA ATC A-3′) of Astrin and Stüben (2008). Each PCR reaction mix (total volume of 12.5 μL) contained 1 μL template DNA, 0.2 m$_M$ dNTPs, 1× PCR buffer, 0.5 μM of each primer, 0.025 U μL^{-1} Hotmaster *Taq*-polymerase (5 PRIME GmbH, Hamburg, Germany) and the rest water. PCR cycle conditions were as follows: initial denaturation for 1 min at 94 °C, followed by 36 cycles of 2 min at 94 °C (denaturation), 20 s at 50 °C (annealing) and 30 s at 65 °C (elongation), and a final elongation step for 5 min at 65 °C.

For some of the acanthocephalans, the nuclear ITS1-5.8S-ITS2 loci were additionally investigated to validate COI results using a newly developed *Polymorphus*-specific primer pair pmF (5′-CCT CAC GGT AAT TCT ATC AG TC-3′) and pmR (5′-CGC AAT CGT GTC ATC TCA GT-3′). The same PCR reaction mix volumina were used, and the following PCR cycle conditions applied: initial denaturation for 5 min at 94 °C, followed by 37 cycles of 40 s at 94 °C (denaturation), 35 s at 56 °C (annealing) and 35 s at 68 °C (elongation), and a final elongation step for 5 min at 68 °C.

Prior to the sequencing of the COI and the nuclear fragment, an ExoI/FastAP purification step was performed. For this purpose, 9 μL of each PCR product was mixed with 1 μL FastAP (1 U μL^{-1}) and 0.5 μL ExoI (20 U μL^{-1}), both from Thermo Fisher Scientific (Schwerte, Germany). The PCR products were enzymatically purified at 37 °C for 25 min and at 85 °C for 15 min. The PCR products were sequenced at GATC Biotech AG (Köln, Germany) using the respective PCR primer pair.

Genetic analyses

Three separate alignments of the resulting host (COI) and parasite sequences (COI and ITS1) were created in Geneious v.6.0.5 (Kearse *et al.* 2012) using the Muscle plug-in with three iterations. Alignments were trimmed by first removing the primer sequences and second using the GBLOCKS web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), thereby further accounting for and automatically removing poorly aligned flanking regions (e.g. resulting from ambiguities or missing data). Final alignments had a length of 658 bp (COI hosts), 432 bp (COI parasites) and 311 bp (ncDNA loci), with the latter encompassing the 3′-region of the ITS1 locus, the complete 5.8S rRNA gene (162 nucleotides) and a minor fragment of the 5′-end of the ITS2 locus (25 nucleotides, excluded from downstream analyses) as identified by comparison with the annotated ITS1-5.8S-ITS2 *P. minutus* sequence available in NCBI GenBank (AY532067).

The number of potential cryptic *Polymorphus* cf. *minutus* species was calculated by the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.* 2012). For this purpose, all available COI sequences (excluding pseudogenes) with a length >500 bp were retrieved from GenBank as on 08.08.2017 (*n* = 322). Sequences were aligned using the Muscle-plugin of Geneious (three iterations) and trimmed to the 655 bp (for acanthocephalans) and 658 bp (for gammarids) long Folmer-fragment. The Polymorphidae COI alignment was combined with the COI parasite alignment of this study. Identical sequences were deleted using the web application ElimDupes

(https://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html), resulting in 264 unique Polymorphidae sequences from NCBI and 126 from this study. Because sequences had unequal lengths, the COI consensus alignment (*n* = 390) was further trimmed using the GBLOCKS web server to a total alignment length of 423 bp. The ABGD method was performed on this alignment using the following final setting: Pmin 0.001; Pmax 0.1; Steps: 50; using a Kimura K80 model (ratio 1.5). A Neighbour-Joining (NJ) tree was constructed for visualization of the ABGD clusters using the Geneious Tree Builder (tree building method: NJ; genetic distance model: Jukes–Cantor; 500 bootstrap replicates).

Amphipod hosts were identified by comparing the obtained COI sequences with the Barcode of Life Datasystem reference library (BOLD; Ratnasingham and Hebert, 2007).

Parasite COI sequences of length <450 bp were used for identification purpose only and excluded from subsequent intraspecific genetic diversity analyses. Haplotype networks were calculated in PopArt (Leigh and Bryant, 2015) using the TCS option (Clement *et al.* 2000). Haplotype and nucleotide COI diversity were calculated in DnaSP v5 (Librado and Rozas, 2009). Intraspecific genetic distances were calculated with MEGA6 (Tamura *et al.* 2013). The ITS1-5.8S alignment was used for visual identification of nuclear alleles.

Results

Amphipod and parasite sampling

At each site, a variable number of one to 33 *Polymorphus*-infected amphipods was available for genetic analyses, largely depending on local prevalences (see Supplementary Table S1). Sequencing of the intermediate hosts confirmed the morphological identification, except cases when no amphipod samples were available for molecular analyses. At sites 25 and 26, *Echinogammarus* specimens were not identified to species level and are treated as *Echinogammarus* sp. (either *E. ischnus* or *E. trichiatus*, the only described *Echinogammarus* spp. in Germany besides *E. berilloni* that can be identified easily). *Polymorphus* infections were found in at least five amphipod species (*Echinogammarus berilloni*, *Echinogammarus* sp., *Gammarus fossarum* type B, *G. pulex*, *G. roeselii*). At two locations, more than one *Polymorphus*-infected amphipod species were present: site 5 (Lippe, *G. roeselii* and *E. berilloni*) and site 25 (Lauter, *Echinogammarus* sp. and *G. fossarum*). Usually, intermediate hosts were infected by a single cystacanth. However, two and three *Polymorphus* individuals occurred in single intermediate host specimens at four sites: site 4 (three cystacanths in *E. berilloni*), site 11 (three in *G. fossarum*), site 17 (2× two in *G. fossarum*) and site 20 (3× two in *G. fossarum*). At five sites (sites 1, 2, 22, 25, 27), amphipod populations were additionally infected by the acanthocephalan parasite *Pomphorhynchus laevis* (see Supplementary Table S1 for details).

Interspecific genetic diversity of the parasite

All individual COI (*n* = 145), ITS1 (66) and 5.8S (66) parasite sequences were deposited in the BOLD project 'PACDE' with BOLD identifiers PACDE001-18 to PACDE178-18. Sequencing of the parasite COI locus revealed the presence of three cryptic *Polymorphus* lineages, as of now referred to as *Polymorphus* type 1-3 (PspT1-3). Molecular species delimitation *via* ABGD resulted in 30 clusters for the investigated COI sequences of the full Polymorphidae dataset retrieved from GenBank, providing further support for the three *Polymorphus* types (Fig. 2). Among those, specimens of PspT3 demonstrated a maximum sequence identity of 99% to the COI (GenBank accession no.
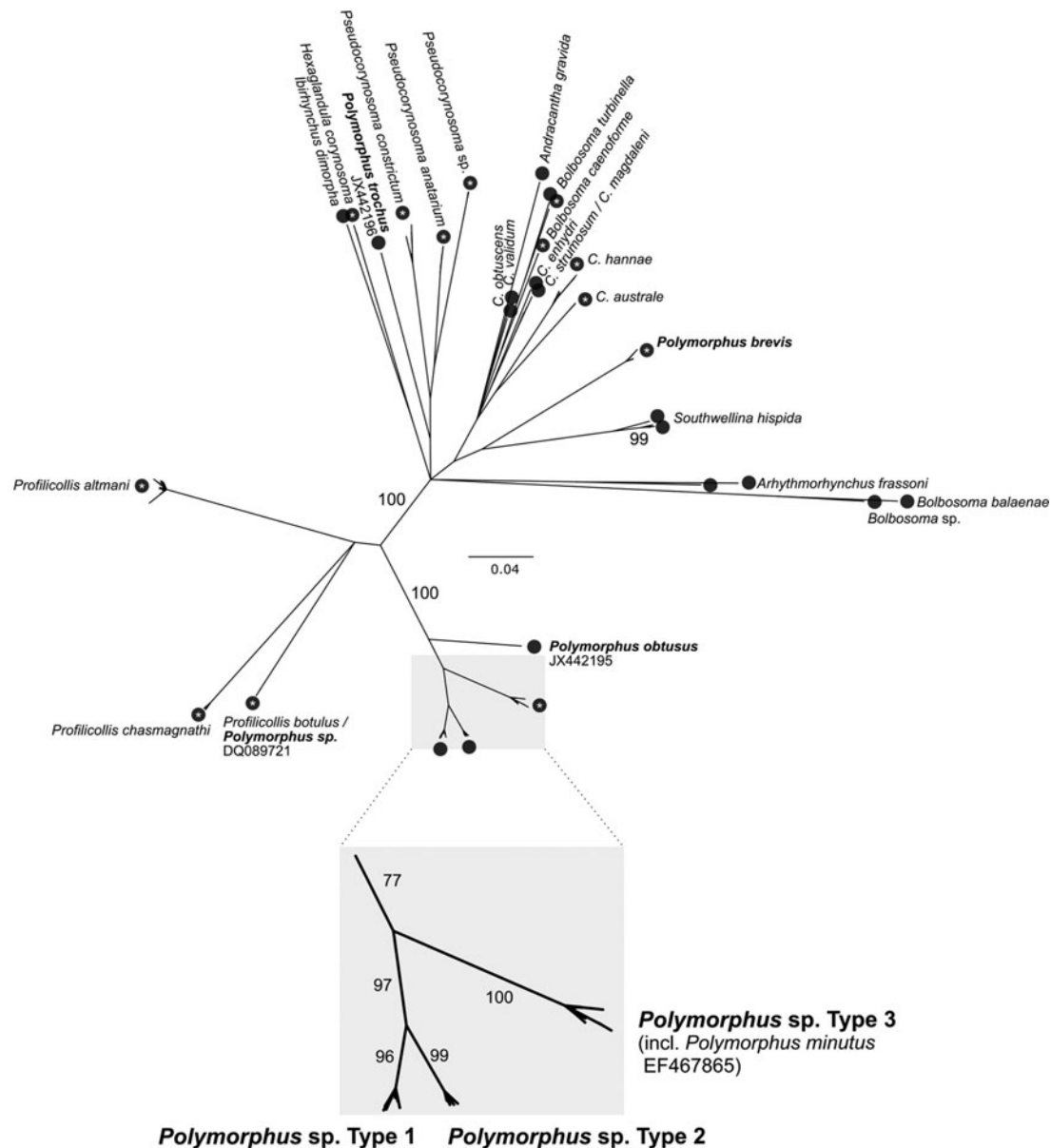
Fig. 2. Molecular Species Delimitation. COI Neighbour-Joining tree for visualization of molecular species delimitation results. Black circles represent clusters proposed by the ABGD method. White asterisks within the circles indicate a 100% bootstrap support for the respective cluster. Further bootstrap supports are provided at the branches.

EF467865.1) and ITS sequence (AY532067.1) of *P. minutus* available in GenBank, respectively (originating from *G. pulex* from Dijon, France as published in García-Varela and Pérez-Ponce de León, 2008 and García-Varela *et al.* 2005). PspT1 was most similar (99%) to JF803287.1 in GenBank (from *G. fossarum* in Switzerland, published in Westram *et al.* 2011).

Based on the investigated COI locus, the three *Polymorphus* types are genetically differentiated by at least 3.5% (PspT1/PspT2), with a maximum interspecific distance of 10.9% between specimens of PspT1 and PspT3 (Table 1). The pattern revealed by the nuclear ITS1 marker is in full congruence with the three mitochondrial COI lineages observed: PspT1 and PspT2 both have one specific ITS1 allele each, with only PspT3 possessing two but likewise specific ITS1 alleles, which are differentiated by one mutation (Fig. 3). The 5.8S rDNA was completely identical for all investigated specimens. For 35 specimens (8× PspT1, 6× PspT2, 21× PspT3), we obtained both the mitochondrial COI and the nuclear ITS1-5.8S fragment, linking the different datasets (Supplementary Table S1 and Supplementary Fig. S1).

Intraspecific genetic diversity

Maximum values of intraspecific genetic distances vary from 1.4% in PspT2 to 2.3% in PspT3 (Table 1). Nucleotide diversity for all three *Polymorphus* types is moderate (π: 0.004–0.007), whereas haplotype diversity is very high (hd: 0.80–0.92). The highest number of haplotypes is observed in PspT3 ($H = 22$), followed by PspT2 (21) and PspT1 (12). The most frequently observed haplotypes are centred within the haplotype networks, which show characteristic star-like patterns (Fig. 4). Since the number of investigated parasite specimens for each *Polymorphus* type were inconsistent for the different localities, only specimen-based haplotype diversity is depicted and no further site-specific analyses were performed (Supplementary Table S1).

Intermediate host specificity

The three *Polymorphus* types are widely distributed and demonstrate a high level of intermediate host specificity (Fig. 1).

Table 1. Genetic distances, nucleotide and haplotype diversity based on the COI fragment of the *Polymorphus* types

	PspT1 (%)	PspT2 (%)	PspT3 (%)	*n*	*S*	*π*	*H*	Hd
PspT1	0.0–1.9			48	21	0.00435 (s.d.: 0.00067)	21	0.836 (s.d.: 0.051)
PspT2	3.5–5.1	0.0–1.4		52	13	0.00364 (s.d.: 0.00041)	12	0.801 (s.d.: 0.041)
PspT3	8.3–10.9	8.6–10.6	0.0–2.3	46	29	0.00737 (s.d.: 0.00076)	22	0.919 (s.d.: 0.026)

PspT1 was exclusively found in *G. fossarum* type B (10 sites). PspT2 was only found in *Echinogammarus* sp./*E. berilloni* (eight sites). PspT3 was restricted to *G. pulex* (six sites) and *G. roeselii* (five sites). At sites where two *Polymorphus* types were co-occurring, each of the two types was found only in a single intermediate host species: site 5 (Klostermersch, PspT2 in *E. berilloni* and PspT3 in *G. roeselii*) and site 25 (Lauter, PspT1 in *G. fossarum* and PspT2 in *Echinogammarus* sp.).

Discussion

In the present study, we investigated the genetic diversity of the acanthocephalan endoparasite *P. minutus* (Goeze, 1782). Three cryptic species were detected (*Polymorphus* type 1–3; PspT1-3), which are congruently supported by mitochondrial (COI) and nuclear (ITS1) data. Our results further indicate that all three potential species are specific for their intermediate amphipod host genus or species.

Cryptic species are a common phenomenon among the Acanthocephala (Wayland, 2010; Pérez-Ponce de León and Poulin, 2017). Even for our target organism, *P. minutus*, there have been indications of different intermediate host specificities (Hynes and Nicholas, 1958) that might be attributable to cryptic *Polymorphus* species. Recent results also indicate morphological differences between isolates of *P. minutus* (Zittel and Taraschewski, unpublished results); therefore the PspT1-3 might turn out to be pseudo-cryptic. However, until now, *P. minutus* was considered the only species of its genus in Central Europe frequently parasitizing amphipod intermediate hosts that are commonly used for ecotoxicological (summarized in Sures *et al.* 2017) and behavioral studies (see, e.g., Helluy, 2013 and references therein). In the light of our results of three cryptic and potentially intermediate-host specific *Polymorphus* cf. *minutus* lineages, the comparison of findings between studies based on different intermediate host–parasite systems has to be treated with caution. However, the most frequently investigated amphipods studied for the influence of parasite infections – *G. roeselii* and

G. pulex (e.g. Bauer *et al.* 2005; Médoc *et al.* 2006; Lagrue *et al.* 2013) – host the same parasite species (i.e. PspT3) and previous results are thus likely to be comparable.

For the study of parasite–host specificity, the correct identification of the host is crucial. Yet, morphological differentiation of *G. pulex* and *G. fossarum* specimens is particularly challenging, especially when no fully mature, adult individuals are available (Karaman and Pinkster, 1977). Additionally, Chen *et al.* (2015) have previously shown that infected individuals are often smaller and therefore more difficult to identify. Adding even more complexity, *G. fossarum* as well as *G. pulex* morphospecies are both known to comprise a high degree of cryptic lineages/species, sometimes showing even different ecological requirements (Lagrue *et al.* 2014; Weiss *et al.* 2014; Grabner *et al.* 2015; Eisenring *et al.* 2016). DNA barcoding of intermediate hosts and parasites may help to circumvent potential future misinterpretations based on misidentified host–parasite relationships, as both 'cryptic dimensions' have to be correctly assessed.

Nearly one-third of all infected intermediate host specimens detected in this study belonged to *G. fossarum* type B, being solely infected by PspT1, indicating a specific parasite–host relationship. This assumption is supported by a previous study on *Polymorphus* infections of *Gammarus* spp. populations in Switzerland (Westram *et al.* 2011). In the latter study, a total of 58 populations have been investigated with 13 populations being infected by *Polymorphus* cf. *minutus*. Comparing the deposited ITS1 sequence from Westram *et al.* (2011) to our data, we can identify the Swiss *Polymorphus* lineage as PspT1 (with 99% sequence identity to JF803287). Only a single specimen of *G. fossarum* type A, but to the large majority *G. fossarum* type B were infected by PspT1, although eight *G. pulex* populations were investigated in the same study, of which two occurred in sympatry with PspT1-infected *G. fossarum* type B populations (see supplementary material of Westram *et al.* 2011). The consistent finding of the same intermediate host–parasite relationship (i.e. PspT1 exclusively infecting *G. fossarum* type B) for another geographical region further strengthens the assumption of intermediate host
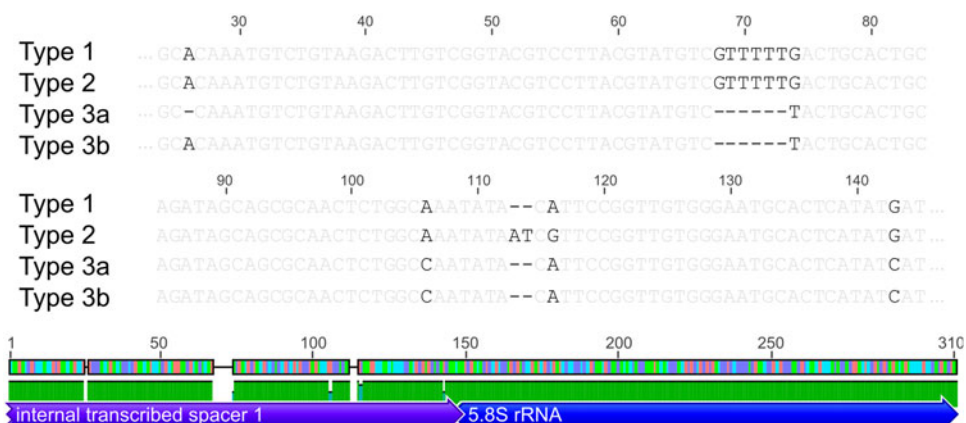

Fig. 3. Nuclear ITS1 alleles. Indication of nuclear ITS1 alleles for the three *Polymorphus* sp. types. Base pair differences are highlighted in black. *Polymorphus* sp. types 1 and 2 possess one ITS1 allele each, whereas *Polymorphus* sp. type 3 demonstrates two, distinguished by one mutation. The 5.8S rDNA was completely identical for all investigated specimens. Figure elements are partly used from Geneious v.6.0.5 (Kearse *et al.* 2012).
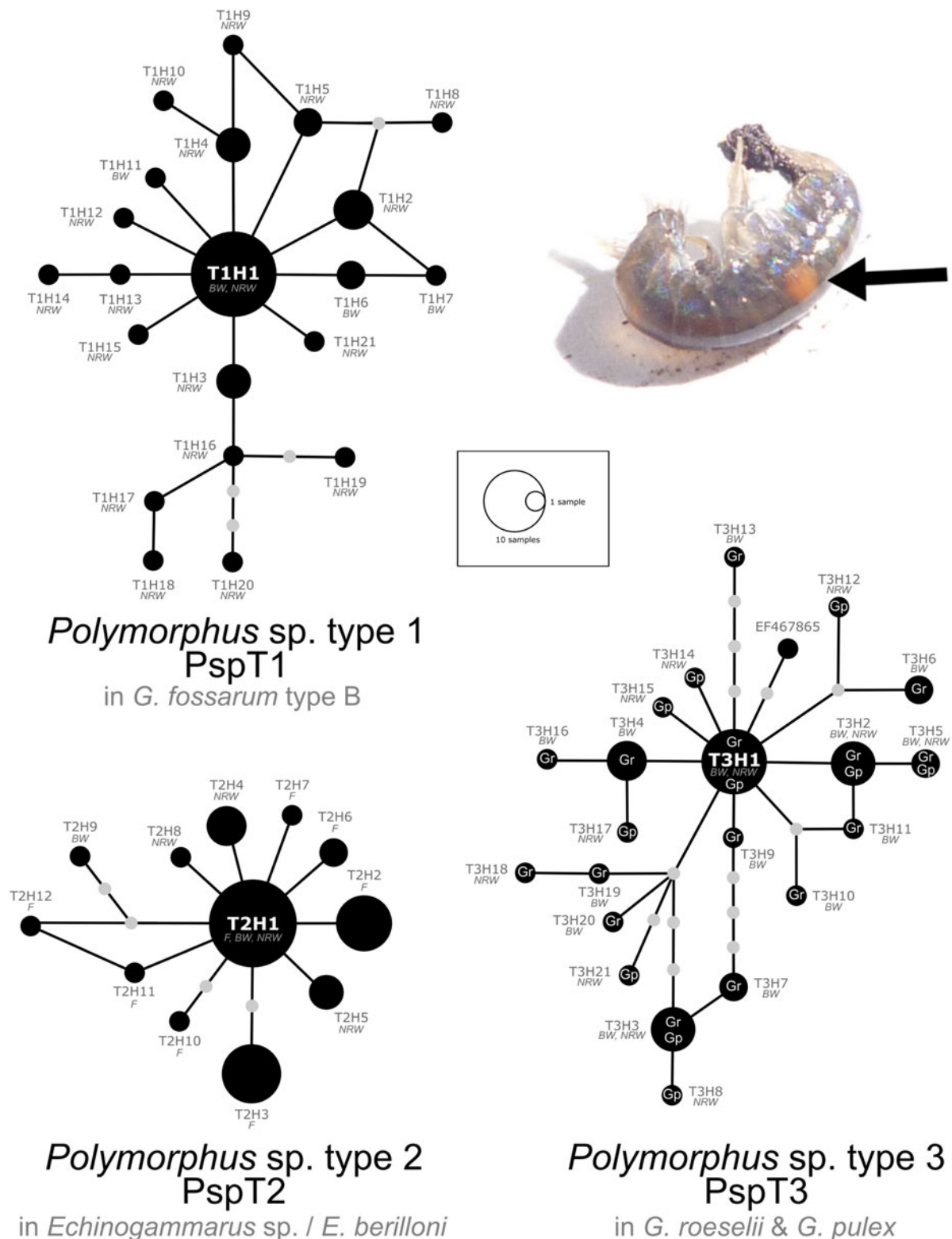
Fig. 4. COI haplotype networks for the three *Polymorphus* sp. types are indicated. The size of the circles is proportional to the frequency of the respective haplotype encountered in each of the three datasets. Grey dots represent hypothetical haplotypes not sampled within this study. Lines between (hypothetical) haplotypes indicate one mutational step. Spatial data for all haplotypes are given (NRW: North Rhine-Westphalia; BW: Baden-Württemberg; F: France). For PspT3, the distribution of the individual haplotypes in the two hosts, *G. pulex* (Gp) and *G. roeselii* (Gr) is indicated. The arrow points to a *Polymorphus* sp. cystacanth in its amphipod intermediate host.

specificity of PspT1. In the present study, PspT2 was detected exclusively in *Echinogammarus* spp. both from Seine and Rhine tributaries, which suggests that this is at least a genus-specific parasite–host association. PspT3 also seems to be less restricted to a single host species, as it was found in both *G. pulex* and *G. roeselii*, which is in accordance with the hypothesis of Kennedy

(2006) that certain acanthocephalan species evolve with multiple intermediate host species.

Besides the results presented here, previous studies provide further evidence for the potential intermediate host specificity of the different *Polymorphus* types. Jacquin *et al.* (2014) detected *Polymorphus* sp. infections in *E. berilloni*, but not in the

co-occurring *G. pulex*. In a study conducted in Ireland, *Polymorphus* sp. was detected in native *G. duebeni celticus* (in 2/70 and 9/65 specimens, respectively), but not in co-occurring non-native *G. tigrinus* (43 investigated specimens) and *G. pulex* (30 specimens) from the same sites (Dunn and Dick, 1998), taking into account that the number of specimens might have been too low to detect low prevalence infections.

Besides the amphipod species mentioned above, *Polymorphus* infections have also been observed in *Echinogammarus stammeri* (Dezfuli and Giari, 1999), *Echinogammarus tibaldii* (Dezfuli *et al.* 2008), *Gammarus lacustris* (Hynes and Nicholas, 1958), *Gammarus oceanicus*, *Gammarus salinus* and *Gammarus zaddachi* (Lehtonen and Hario, 1996) in different European countries. This diversity of intermediate hosts suggests that even more species within the *Polymorphus* cf. *minutus* complex might exist than those discovered in the present study.

The reason for intermediate host specificity in acanthocephalans might be seen in the adaptation to the invertebrate host immune response. For example, Hynes and Nicholas (1958) found large numbers of dead larvae of *P. minutus* at the outer surface of the gut of experimentally infected amphipods when eggs of parasites were used that originated from cystacanths isolated from another amphipod species. This is in line with findings for other acanthocephalans showing that the migration of the parasite through the gut wall of the intermediate host in the early phase of infection is most crucial for survival (Taraschewski, 2000).

Based on our data, we can only speculate on the specificity of the three *Polymorphus* sp. types for their definitive hosts. Numerous bird species were listed as hosts for *P. minutus* (e.g. Lühe, 1911), while anatid ducks and goose are probably the most common definitive hosts. Furthermore, there are some reports of *Polymorphus* spp. from water-related rodents such as water shrews or muskrats (summarized in Platt, 1978). The question, if the three *Polymorphus* types identified in the present study show some sort of definitive host specificity is highly relevant for the understanding of the biology of this genus. Either each of the respective *Polymorphus*-types will develop only in a specific definitive host species (due to species-specific strategies overcoming the hosts immune systems), or if they colonize the same definitive host individual, some mechanism of reproductive isolation must exist (e.g. by preferring different sections of the host gut, or by showing different seasonality patterns). Due to a lack of *Polymorphus*-specimens from definitive hosts, we can only speculate here. Also, we are not able to trace back which *Polymorphus* type actually refers to *P. minutus* (Goeze, 1782), initially described as *Echinorhynchus minutus*, potentially from *Melanitta fusca* and *Turdus merula* (pages 164 & 165 in Goeze, 1782) in Germany. It is questionable if this will ever be possible due to the lack of morphological characters given in these early studies (Smales, 2014).

The present study also provides evidence for at least one non-native acanthocephalan in Germany: PspT2 was detected only in *Echinogammarus*, most of which were *E. berilloni*. This amphipod is an established, non-native species in Germany that originated from the Mediterranean region (France and Spain) and that was recorded for the first time in Germany in 1924 (Tittizer *et al.* 2000). As PspT2 was not found in native amphipods, it seems likely that *E. berilloni* introduced this *Polymorphus* type to Central Europe. Also, PspT3 might be a non-native parasite that was brought to Central Europe a few hundred years ago by the invasion of *G. roeselii* from the Balkan region (Grabowski *et al.* 2017). If this scenario is true, PspT3 must have colonized *G. pulex* as an additional host species. Nevertheless, it seems more likely that *G. pulex* is the native host for PspT3 and *G. roeselii* was colonized later, given that PspT3: (a) occurs in an indigenous amphipod species (*G. pulex*) and (b) is the genetically

most diverse parasite lineage in our dataset (in terms of nucleotide and haplotype diversity), not pointing to any severe decline in genetic diversity due to potential historical bottleneck events during invasion processes. Recently, evidence for another non-native acanthocephalan in the Rhine system was presented (Hohenadler *et al.* 2017). In the case reported by the latter authors, the fish infecting acanthocephalan *Pomphorhynchus laevis* was most likely introduced by invasive goby species that can be used as paratenic hosts for this parasite. In addition, the eoacanthocephalan *Paratenuisentis ambiguus* is also a non-native species (Taraschewski *et al.* 1987), originating from North America where it was described from *Gammarus tigrinus* as an intermediate host and the American eel as the definitive host. Interestingly, following the transplantation of *G. tigrinus* into the rivers Weser and Werra in the late 1950s, this acanthocephalan started to migrate into different rivers and accepted the European eel as a new definitive host, where it reached high prevalences and intensities (Sures and Streit, 2001). In contrast to its final host switch, *P. ambiguus* was never described from other gammarid species than *G. tigrinus*, which also suggests a clear intermediate host specificity. Taken together, these examples show that recent species introductions are common for acanthocephalans, but the mechanisms of invasion vary.

In conclusion, we provide consistent support for the existence of at least three cryptic parasite species within *Polymorphus* cf. *minutus*, which very likely demonstrate a high degree of host specificity for their amphipod intermediate hosts. The immunological adaptation of the acanthocephalan to the intermediate host and the biochemical alterations caused by the parasite (Taraschewski, 2000; Helluy, 2013) might be a possible driver of this diversification. To correctly assess (intermediate) host–parasite relationships in the future, the application of routine DNA barcoding is highly encouraged (but see Pérez-Ponce de León and Nadler, 2010; Alcántar-Escalera *et al.* 2013). According to our results, we also suggest reviewing the taxonomy of *Polymorphus* spp. in Europe including adult stages to further clarify, whether the three *Polymorphus* types identified in the present study are valid species also differing in definitive host specificity and morphology.

References

Albrecht H, Ehlers U and Taraschewski H (1997) Syncytial organisation of acanthors of *polymorphus minutus* (palaeacanthocephala), *neoechinorhynchus rutile* (eoacanthocephala), and *moniliformis moniliformis* (archiacanthocephala) (acanthocephala). *Parasitology Research* **83**, 326–338.

Alcántar-Escalera FJ, García-Varela M, Vázquez-Domínguez E and Pérez-Ponce de León G (2013) Using DNA barcoding to link cystacanths and adults of the acanthocephalan *polymorphus brevis* in central Mexico. *Molecular Ecology Resources* **13**, 1116–1124.

Astrin JJ and Stüben PE (2008) Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). *Invertebrate Systematics* **22**(5), 503–522.

Bauer A, Haine ER, Perrot-Minnot M-J and Rigaud T (2005) The acanthocephalan parasite *Polymorphus minutus* alters the geotactic and clinging behaviours of two sympatric amphipod hosts: the native *Gammarus pulex* and the invasive *Gammarus roeseli*. *Journal of Zoology* **267**, 39–43.

Bollache L, Gambade G and Cézilly F (2001) The effects of two acanthocephalan parasites, *Pomphorhynchus laevis* and *Polymorphus minutus*, on pairing success in male *Gammarus pulex* (Crustacea: Amphipoda). *Behavioral Ecology and Sociobiology* 49, 296–303.

Chen H-Y, Grabner DS, Nachev M, Shih H-H and Sures B (2015) Effects of the acanthocephalan *Polymorphus minutus* and the microsporidian *Dictyocoela duebenum* on energy reserves and stress response of cadmium exposed *Gammarus fossarum*. *PeerJ* 3, e1353.

Clement M, Posada DCKA and Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9(10), 1657–1659.

Crompton DWT and Lee DL (1965) The fine structure of the body wall of *Polymorphus minutus* (Goeze, 1782) (Acanthocephala). *Parasitology* 55, 357–364.

Dezfuli BS and Giari L (1999) Amphipod intermediate host of *Polymorphus minutus* (Acanthocephala), parasite of water birds, with notes on ultrastructure of host-parasite interface. *Folia Parasitologica* 46, 117–122.

Dezfuli BS, Lui A, Giovinazzo G and Giari L (2008) Effect of Acanthocephala infection on the reproductive potential of crustacean intermediate hosts. *Journal of Invertebrate Pathology* 98, 116–119.

Dunn AM and Dick JTA (1998) Parasitism and epibiosis in native and non-native gammarids in freshwater in Ireland. *Ecography* 21, 593–598.

Eggers TH and Martens A (2001) A key to the freshwater Amphipoda (Crustacea) of Germany. *Lauterbornia* 42, 1–68.

Eggers TH and Martens A (2004). Additions and Corrections "to A key to the freshwater Amphipoda (Crustacea) of Germany". *Lauterbornia* 50, 1–13.

Eisenring M, Altermatt F, Westram AM and Jokela J (2016) Habitat requirements and ecological niche of two cryptic amphipod species at landscape and local scales. *Ecosphere* 7(5), e01319.

García-Varela M and Pérez-Ponce de León G (2008) Validating the systematic position of *Profilicollis* Meyer, 1931 and *Hexaglandula* Petrochenko, 1950 (Acanthocephala: Polymorphidae) using cytochrome c oxidase (Cox 1). *The Journal of Parasitology* 94, 212–217.

García-Varela M, Aznar FJ, Pérez-Ponce de León G, Piñero D and Laclette JP (2005) Molecular phylogeny of *Corynosoma* Lühe, 1904 (Acanthocephala), based on 5.8S and internal transcribed spacer sequences. *The Journal of Parasitology* 91, 345–352.

Goeze JAE (1782). *Versuch Einer Naturgeschichte der Eingeweidewürmer Thierischer Körper*. Blankenburg, Germany: Pape.

Grabner DS, Weigand AM, Leese F, Winking C, Hering D, Tollrian R and Sures B (2015) Invaders, natives and their enemies: distribution patterns of amphipods and their microsporidian parasites in the Ruhr Metropolis, Germany. *Parasites & Vectors* 8(1), 419.

Grabowski M, Mamos T, Bącela-Spychalska K, Rewicz T and Wattier RA (2017) Neogene paleogeography provides context for understanding the origin and spatial distribution of cryptic diversity in a widespread Balkan freshwater amphipod. *PeerJ* 5, e3016. https://doi.org/10.7717/peerj.3016

Greef R (1864) Untersuchungen über den Bau und die naturgeschichte von echinorhynchus miliarius zenker (E. Polymorphus). *Archiv für Naturgeschichte* 30(1), 98–140.

Haine ER, Boucansaud K and Rigaud T (2005) Conflict between parasites with different transmission strategies infecting an amphipod host. *Proceedings. Biological sciences/The Royal Society* 272, 2505–2510.

Helluy S (2013) Parasite-induced alterations of sensorimotor pathways in gammarids: collateral damage of neuroinflammation? *The Journal of Experimental Biology* 216, 67–77.

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. https://doi.org/10.1007/s10530-017-1527-9

Hynes HBN and Nicholas WL (1958) The resistance of *Gammarus* spp. to infection by *Polymorphus minutus* (Goeze, 1782) (Acanthocephala). *Annals of Tropical Medicine and Parasitology* 52, 376–383.

Hynes HBN and Nicholas WL (1963) The importance of the acanthocephalan *Polymorphus minutus* as a parasite of domestic ducks in the United Kingdom. *Journal of Helminthology* 37, 185–198.

Itämies J, Valtonen ET and Fagerholm HP (1980) *Polymorphus minutus* (Acanthocephala) infestation in eiders and its role as a possible cause of death. *Annales Zoologici Fennici* 17(4), 285–289.

Jacquin L, Mori Q, Pause M, Steffen M and Medoc V (2014) Non-specific manipulation of gammarid behaviour by P. minutus parasite enhances their predation by definitive bird hosts. *PLoS ONE* 9, 1–11.

Karaman G and Pinkster S (1977) Freshwater *Gammarus* species from Europe, North Africa and adjacent regions of Asia (Crustacea-Amphipoda). Part I. Gammarus pulex-group and related species. *Bijdragen tot de Dierkunde* 47, 1–97.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P and Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28(12), 1647–1649.

Kennedy CR (2006). *Ecology of the Acanthocephala*. Cambridge, UK: Cambridge University Press.

Lagrue C, Güvenatam A and Bollache L (2013) Manipulative parasites may not alter intermediate host distribution but still enhance their transmission: field evidence for increased vulnerability to definitive hosts and non-host predator avoidance. *Parasitology* 140, 258–265.

Lagrue C, Wattier R, Galipaud M, Gauthey Z, Rullmann JP, Dubreuil C, Rigaud T and Bollache L (2014) Confrontation of cryptic diversity and mate discrimination within Gammarus pulex and *Gammarus fossarum* species complexes. *Freshwater Biology* 59(12), 2555–2570.

Lehtonen JT and Hario M (1996) The role of different gammarid communities in transmission of *Polymorphus minutus* (Acanthocephala) to eiders. *Bulletin of the Scandinavian Society for Parasitology* 6(2), 122.

Leigh JW and Bryant D (2015) Popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6(9), 1110–1116.

Librado P and Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25(11), 1451–1452.

Lühe M (1911). Acanthocephalen. In Brauer A (ed). *Die Süsswasserfauna Deutschlands 16*. Jena, Germany: G. Fischer, pp. 1–60.

Médoc V, Bollache L and Beisel J-N (2006) Host manipulation of a freshwater crustacean (Gammarus roeseli) by an acanthocephalan parasite (*Polymorphus minutus*) in a biological invasion context. *International Journal for Parasitology* 36, 1351–1358.

Nadler SA and Pérez-Ponce de León G (2011) Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. *Parasitology* 138, 1688–1709.

Pérez-Ponce de León G and Nadler SA (2010) What we don't recognize can hurt us: a plea for awareness about cryptic species. *Journal of Parasitology* 96, 453–464.

Pérez-Ponce de León G and Poulin R (2017) An updated look at the uneven distribution of cryptic diversity among parasitic helminths. *Journal of Helminthology*, 1–6. doi: 10.1017/S0022149X17000189

Platt TR (1978) Research note-a report of polymorphus paradoxus (Acanthocephala) in microtus pennsylvanicus from hastings lake, Alberta (Canada). *Proceedings of the Helminthological Society of Washington* 45(2), 255.

Puillandre N, Lambert A, Brouillet S and Achaz G (2012) ABGD, automatic barcode gap discovery for primary species delimitation. *Molecular Ecology* 21(8), 1864–1877.

Ratnasingham S and Hebert PD (2007) BOLD: the barcode of life data system (http://www.barcodinglife.org). *Molecular Ecology Resources* 7(3), 355–364.

Schmidt GD (1985). Development and life cycles. In Crompton DWT and Nickol BB (eds). *Biology of the Acanthocephala*. Cambridge, UK: Cambridge University Press, pp. 273–286.

Selbach C, Soldánová M, Georgieva S, Kostadinova A and Sures B (2015) Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the 'Diplostomum mergi' species complex. *Parasites and Vectors* 8, 300.

Smales L (2014). Acanthocephala. In Schmidt-Rhaesa A (ed) *Handbook of Zoology, Gastrotricha, Cycloneuralia and Gnathifera*, vol. **3**, pp. 313–332. Berlin, Germany: de Gruyter.

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

Sures B (2014). Ecology of the acanthocephala. In Schmidt-Rhaesa A (ed) *Handbook of Zoology, Gastrotricha, Cycloneuralia and Gnathifera*, vol. **3**. Berlin, Germany: de Gruyter, pp. 337–344.

Sures B and Streit B (2001) Eel parasite diversity and intermediate host abundance in the River Rhine, Germany. *Parasitology* 123, 185–191.

Sures B, Nachev M, Selbach C and Marcogliese DJ (2017) Parasite responses to pollution: what we know and where we go in "Environmental Parasitology". *Parasites & Vectors* 10, 65.

Tain L, Perrot-Minnot M-J and Cézilly F (2006) Altered host behaviour and brain serotonergic activity caused by acanthocephalans: evidence for specificity. *Proceedings of Biological Sciences/The Royal Society* 273, 3039–3045.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**(12), 2725–2729.

Taraschewski H (2000) Host-parasite interactions in acanthocephala: a morphological approach. *Advances in Parasitology* **46**, 1–179.

Taraschewski H, Moravec F, Lamah T and Anders K (1987) Distribution and morphology of two helminths recently introduced into European eel populations: *Anguillicola crassus* (Nematoda, Dracunculoidea) and *Paratenuisentis ambiguus* (Acanthocephala, Tenuisentidae). *Diseases of Aquatic Organisms* **3**, 167–176.

Tittizer T, Schöll F, Banning M, Haybach A and Schleuter M (2000) Aquatic neozoan invertebrates in the inland water ways of the Federal Republic of Germany. *Lauterbornia* **39**, 1–72.

Wayland MT (2010) Proboscis profiler: a tool for detecting acanthocephalan morphotypes. *Systematic Parasitology* **76**, 159–167.

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.

Weiss M, Macher JN, Seefeldt MA and Leese F (2014) Molecular evidence for further overlooked species within the *Gammarus fossarum* complex (Crustacea: Amphipoda). *Hydrobiologia* **721**(1), 165–184.

Westram AM, Baumgartner C, Keller I and Jokela J (2011) Are cryptic host species also cryptic to parasites? Host specificity and geographical distribution of acanthocephalan parasites infecting freshwater *Gammarus*. *Infection, Genetics and Evolution* **11**, 1083–1090.