Research Paper

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Helminth endoparasites of the smooth newt *Lissotriton vulgaris*: linking morphological identification and molecular data

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Abstract

The helminth endoparasites of many European amphibian species are often known exclusively from morphological descriptions. A molecular library of DNA sequence data linked to morphological identifications is still in its infancy. In this paper, we aim to contribute to such a library on the smooth newt *Lissotriton vulgaris,* the intermediate and definitive host of 31 helminth parasites, according to evidence published so far. Newts (*n* = 69) were collected at two study sites in western Germany and examined for the presence of helminths. A total of five helminth species were detected in 56 (81%) of the newts, but only one or two species infected a single host. Four out of five helminth species were identified morphologically and based on DNA sequences as *Parastrigea robusta* (metacercariae), *Oswaldocruzia filiformis, Megalobatrachonema terdentatum* (adults and larvae) and *Cosmocerca longicauda*, and the corresponding sequences were provided subsequently. *Oswaldocruzia molgeta* was confirmed to be a junior synonym of *O. filiformis*. Molecular data on a fifth species (a cosmocercid nematode) that could not be identified at species level were added to GenBank. These findings increased the molecular library on morphologically identified smooth newt parasites significantly, from 12 to 15 entries.

Introduction

Smooth newts *Lissotriton vulgaris* inhabit humid and aquatic areas in most of Europe, from sea level to 2150 m above sea level (asl) in the Austrian Alps. A considerable range of studies on newt populations covering most of their geographical range yielded a well-established overview on their helminth endoparasites (Barus & Groschaft, 1962; Vojtková, 1963a, b; Avery, 1971; Vojtek, 1972; Scholz & Moravec, 1990; Caffara *et al.*, 2014). Local diversity rarely exceeds five parasite species within a single host population, whereas a total of 31 different helminth species have been reported to use *L. vulgaris* as host in general, i.e. two acanthocephalans: *Acanthocephalus anthuris, A. ranae*; two cestodes: *Batrachotaenia carpathica* and *Bothriocephalus claviceps*; 14 trematodes (metacercariae or adults): *Alaria alata* (a foodborne human pathogen), *Astiotrema monticelli, A. trituri, Clinostomum complanatum, Diplodiscus subclavatus, Halipegus kessleri, Leptophallus nigrovenosus, Opistioglyphe ranae, Paralepoderma cloacicola, Parastrigea robusta, Pleurogenes claviger, Pleurogenoides medians, Strigea sphaerula* and *S. strigis*; and 13 nematodes: *Agamospirura* sp. larva, *Amphibiocapillaria filiformis, A. tritonispunctati, Anguillicola crassus, C. longicauda, C. ornata, Hedruris androphora, Megalobatrachonema terdentatum, Oswaldocruzia duboisi, O. goezei, O. filiformis, O. molgeta* and *Oxysomatium brevicaudatum* (Lewis, 1928; Grabda, 1959; Barus & Groschaft, 1962; Vojtková, 1963a, b; Avery, 1971; Vojtková & Vojtek, 1972; Moravec & Vojtková, 1975; Bertman & Okulewicz, 1987; Chumak, 1989; Scholz & Moravec, 1990; Ben Slimane *et al.,* 1993; Iskova et al., 1995; Moravec & Skoríková, 1998, Shimalov *et al.,* 2001; Shimalov, 2002; Caffara *et al.*, 2014). Yet, helminth parasite diversity of smooth newts could turn out to be significantly lower because the list comprises taxa within the nematode genera *Amphiobiocapillaria* and *Oswaldocruzia* which are morphologically indistinguishable (Moravec & Vojtková, 1975; Yildirimhan *et al.*, 2005). Reliable taxonomic identification of newt helminths is hampered by the fact that many parasite species have a broad host spectrum (some infest a wide range of anuran, urodele and reptile species) and that subsequent morphological variation among conspecific parasites collected from distinct hosts may lead to erroneous taxonomic decisions (e.g. Moravec & Vojtková, 1975). Moreover, early developmental stages of nematodes and encysted metacercariae in amphibian hosts often do not offer reliable morphological features to distinguish sufficiently among species (Odening, 1967; Petter & Chabaud, 1971; Locke *et al.*, 2011; Patrelle *et al.*, 2015).

Molecular taxonomy aims to use DNA sequence diversity of one or more uniform target genes as a standardized tool, and has been proposed to solve the shortcomings of the

morphological approach in the affiliation of parasites with a lack of morphological diagnostic characters in adults and larval stages that have not yet been characterized (e.g. Besansky *et al.*, 2003; Vanhove *et al.*, 2013). Internal transcribed spacer 2 (ITS2) sequences proved useful for the assignment of Trematoda metacercariae to the family level, but identification at species level failed, due to the absence of sequences obtained from adult specimens, which had been identified morphologically (Chontananarth *et al.*, 2017). Cytochrome *c* oxidase 1 (CO1) sequences distinguished 52 species among 2000 diplostomid metacercariae in North America, Europe and Africa, but only 12 were assignable to described species (Locke *et al.*, 2015). In cyathostomin nematodes, reliable morphological species differentiation is limited to adults (Bredtmann *et al.*, 2017). These study results imply that morphological and molecular datasets are widely unlinked, resulting in a low number of reliably identified helminth species with corresponding sequence data deposited in GenBank. Thus, the real diversity of helminth taxa is still in question (Luque *et al.*, 2017).

DNA sequences of morphologically identified endoparasitic helminths of the smooth newt in Germany are presented in this paper. So far, GenBank provides sequences (mainly the 28S rRNA gene) for only 12 (1 cestode, 8 trematodes, 3 nematodes) out of 31 species known to use *L. vulgaris* as host. At least two of them are predominately fish parasites (*A. crassus, C. complanatum*) being atypically found in newts. The five parasitic helminth taxa infesting *L. vulgaris* in western Germany are presented morphologically, together with their corresponding DNA sequences for future molecular identification.

Materials and methods

Collection and examination of newts

Adult *L. vulgaris* were collected from two localities at the former military training area Schmidtenhöhe near Koblenz (Rhineland-Palatinate, Germany) (Sinsch & Breuer, 2018): (1) 44 males and 5 females in a permanent eutrophic pond (50.347°N, 7.674°E, 333 m asl; 10 June 2016, 26 April, 31 May and 21 June 2017) and (2) 20 males in a temporarily water-filled ditch (50.346°N, 7.644°E, 279 m asl; 16 June 2016). All specimens were sacrificed immediately after collection and the body cavity, digestive tract, lungs, kidneys and bladder were subsequently examined macroscopically and microscopically for the presence of helminth parasites. Carcasses of newts were fixed in 10% buffered formalin, and transferred to 70% ethanol for long-term storage. One to three helminth specimens (encysted metacercariae or adult nematodes) of each infected newt were isolated in 1.5-ml reaction tubes with 96% ethanol for DNA extraction, while the remaining helminths were stored in 4% buffered formalin for morphological species identification and documentation. Finally, the newts examined and voucher specimens of helminths were deposited in the collection of the Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany (ZFMK).

Morphological helminth identification

Only encysted trematode metacercariae and larval and adult nematodes were detected. Ellipsoidal cysts were photographed in life under slight coverslip pressure, and axes were measured to the nearest micrometre. Metacercariae were identified based on the keys of Dubois (1968) and Niewiadomska (2002). Features of tentatively identified taxa were compared with those given in specific references on these taxa (e.g. Odening, 1965, 1967). Nematodes were photographed in life and formalin-preserved. Each individual sample was cleared in a drop of lactophenol on a microscope slide, a cover slip was added and it was examined under a compound microscope. The nematodes were identified following the keys of Anderson *et al.* (2009) and Gibbons (2010).

Nucleic acid extractions and polymerase chain reactions

Metacercariae (*n* = 4) were washed twice for 15 min using 1 ml of buffer containing 10 mM Tris–HCl (pH 7.5) and 5 mM EDTA. Subsequently, the samples were processed as described previously (Sitko *et al.*, 2017). The primers targeted nuclear 18S rDNA and ITS1–5.8S–ITS2 loci, and the mitochondrial CO1 locus (table 1). The resulting consensus DNA sequences were submitted to GenBank under accession numbers MF537201–MF537212 (see table 2).

Each ethanol-fixed nematode specimen was incubated, with open caps, at 40°C in a Thermomixer (Eppendorf, Wesseling-Berzdorf, Germany) until the ethanol was completely evaporated. Samples were mixed with 180 μl ATL buffer, 20 μl proteinase K and incubated at 56°C until specimens were completely lysed, and processed following the protocol for DNA purification from tissues of the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was used to amplify parts of 18S rRNA and ITS1 locus using primers S1 and IR8, or partial ITS1, 5.8S rDNA and partial ITS2 locus using primers ITS1 and ITS4, respectively (White *et al.*, 1990; Sinnappah *et al.*, 2001). Polymerase chain reaction (PCR) using *Taq* PCR core kit (Qiagen) was conducted as recommended by the manufacturer. The PCR protocol consisted of an initial cycle at 94°C for 3 min; 40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 3 min; with a last extension step at 72°C for 7 min. The amplicons were separated on a 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

Sequencing and phylogenetic analysis

PCR amplicons were bidirectionally sequenced using the same primers as for PCR. Sequences were assembled by DNA baser (Heracle BioSoft, http://www.dnabaser.com/) and deposited in GenBank (tables 2 and 3). Sequences obtained from metacercariae and sequences of adult Strigeidae that were published previously (Heneberg *et al.*, submitted) were aligned using ClustalW (http://www.genome.jp/tools-bin/clustalw) (gap opening penalty 7, gap extension penalty 2 for both pairwise and multiple alignments, DNA weight matrix IUB, transition weight 0.1). *Cotylurus syrius* served as an outgroup. The alignments were corrected manually for any inconsistencies and the aligned sequences were trimmed to the length covered by all the sequences included. The sequences were trimmed to ITS2 (267 bp, trimmed to nucleotides 1131–1397 of MF537205.2), 18S rDNA (695 bp, corresponds to the full-length MF537201.1) and CO1 (295 bp, trimmed to nucleotides 35–329 of MF537209.1). The trimmed aligned sequences were concatenated and only these concatenated sequences were used for further analyses. Closest matches of nematode sequences were identified by a BLAST search against GenBank entries (Altschul *et al.*, 1990).

Phylogenetic trees were constructed from the alignments using the Maximum Likelihood algorithm with MEGA 5.2 (Tamura

Table 1. Primers used for the amplification and sequencing of nuclear and mitochondrial DNA loci in the helminths examined.

Locus	Primer name	Sequence	Reference
Trematoda			
18S rDNA	C for	ATGGCTCATTAAATCAGCTAT	Routtu *et al.* (2014)
	A rev	TGCTTTGAGCACTCAAATTTG	Routtu *et al.* (2014)
18S rDNA–ITS1–5.8S rDNA–ITS2–28S rDNA	NSF1419/20	ATAACAGGTCTGTGATGCCC	Winnepenninckx *et al.* (1994)
	Dig.5.8SR	CGCAATATGCGTTCAAGATG	Sato *et al.* (2010)
	NC13(ITS2)/F	ATCGATGAAGAACGCAGC	Jacobs *et al.* (1997)
	Dd28SR1	ACAAACAACCCGACTCCAAG	Otranto *et al.* (2007)
CO1	JB3	TTTTTTGGGCATCCTGAGGTTTAT	Bowles *et al.* (1992)
	JB4.5	TAAAGAAAGAACATAATGAAAATG	Bowles *et al.* (1992)
	CO1-R trema	CAACAAATCATGATGCAAAAGG	Mira *et al.* (2005)
Nematoda			
18S rDNA–ITS1	S1	ATTCCGATAACGAACGAGACT	Sinnappah *et al.* (2001)
	IR8	GCTAGCTGCGTTCTTCATCGA	
ITS1–5.8S rDNA–ITS2	ITS1	TCCGTAGGTGAACCTGCGG	White *et al.* (1990)
	ITS4	TCCTCCGCTTATTGATATGC	

et al., 2011). Branch support was calculated based on 1000 bootstraps. The nearest-neighbour interchange served as the maximum likelihood heuristic method of choice to determine the tree inference when the initial tree was formed using a neighbour-joining algorithm. The best model according to the Bayesian information criterion, corrected Akaike information criterion and maximum likelihood values was Hasegawa–Kishino–Yano, with the non-uniformity of evolutionary rates among sites modelled using a discrete Gamma distribution with five rate categories, and by assuming that a certain fraction of sites are evolutionarily invariable (+I). The maximum likelihood method was used to estimate inter- and intraspecific evolutionary divergence in the analysed sequences. The number of base differences per site was calculated by averaging over all sequence pairs between groups (distance) ± SE and by employing a bootstrap procedure at 1000 replicates. The model that was used to estimate inter- and intrasite evolutionary divergence was identical to that used to construct the respective maximum likelihood tree.

Results

The endoparasitic helminth diversity detected in smooth newts collected from the two study sites included one trematode and four nematode species (table 4, fig. 1). 13 (19%) out of the 69 newts inspected were free of helminth parasites. Most newts

Table 2. Sequences of helminth parasites isolated from *L. vulgaris* in the course of the present study. New sequences of *Parastrigea robusta* and the previously published sequences of Strigeidae adults (Heneberg *et al.*, submitted), which were used in the comparative phylogenetic analyses of concatenated sequences in order to allow molecular identification of the new material. The National Centre for Biotechnology Information (NCBI) GenBank accession numbers are indicated.

Specimen	Species, host, sampling site	Locus		
		18S rDNA–ITS1–5.8S rDNA–ITS2–28S rDNA	18S rDNA	CO1
Newly sequenced specimens				
3LF-2836	*Parastrigea robusta* ex *Lissotriton vulgaris*	MF537205.2	MF537201.1	MF537209.1
3LF-2837	*Parastrigea robusta* ex *Lissotriton vulgaris*	MF537206.2	MF537202.1	MF537210.1
3LF-2838	*Parastrigea robusta* ex *Lissotriton vulgaris*	MF537207.2	MF537203.1	MF537211.1
3LF-2839	*Parastrigea robusta* ex *Lissotriton vulgaris*	MF537208.2	MF537204.1	MF537212.1
NCBI accession numbers of previously published sequences that were used in the analyses of concatenated sequences				
3LF-3911	*Parastrigea robusta* ex *Anas platyrhynchos*	MF628100.1	MF628084.1	MF628067.1
3LF-2343	*Strigea strigis* ex *Asio otus*	MF628088.1	MF628074.1	MF628049.1
3LF-2708	*Strigea sphaerula* ex *Acrocephalus arundinaceus*	MF628090.1	MF628073.1	MF628054.1
3LF-3907	*Cotylurus syrius* ex *Cygnus olor*	MF628099.1	MF628083.1	MF628059.1

Table 3. Sequences of helminth parasites isolated from *L. vulgaris* in the course of the present study. New sequences of nematode parasites. NCBI GenBank accession numbers are indicated.

Specimen	Species, host, sampling site	Locus	
		18S rDNA–ITS1	ITS1–5.8S rDNA–ITS2
Newly sequenced specimens			
K13-1601	*Oswaldocruzia filiformis* ex *Lissotriton vulgaris*	MG586086	
ZX3-1701	*Oswaldocruzia filiformis* ex *Lissotriton vulgaris*	MG586087	MG586088
K03-1601	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594354	
K12-1601	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594355	
K12-1602	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594356	
K12-1603	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594357	
K19-1601	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594358	
K19-1602	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594359	
K19-1603	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594360	
K19-1604	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594361	
K19-1605	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594362	
ZX2-1602	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594352	
ZX3-1702	*Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594363	
ZX3-1703	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594364	
ZX4-1604	Larval *Megalobatrachonema terdentatum* ex *Lissotriton vulgaris*	MG594353	
K05-1601	Cosmocercid sp. ex *Lissotriton vulgaris*	MG594348	
K18-1601	*Cosmocerca longicauda* ex *Lissotriton vulgaris*	MG594349	
K18-1602	*Cosmocerca longicauda* ex *Lissotriton vulgaris*	MG594350	
K18-1603	*Cosmocerca longicauda* ex *Lissotriton vulgaris*	MG594351	
NCBI accession numbers of previously published sequences that were used in the analyses of sequences			
KR029782	*Oswaldocruzia filiformis* ex *Rana ridibunda*		KR029782
KR029783	*Oswaldocruzia filiformis* ex *Rana ridibunda*		KR029783
KR029784	*Oswaldocruzia filiformis* ex *Rana ridibunda*		KR029784
JX877697	*Austrostrongylus victoriensis* ex *Wallabia bicolor*		JX877697
AB682701	*Spiculopteragia houdemeri* ex *Capricornis crispus*		AB682701
AB682702	*Spiculopteragia houdemeri* ex *Capricornis crispus*	AB682702	AB682702
AB367797	*Skrjabinagia* sp. ex *Capricornis crispus*		AB367797
LC018444	*Cosmocercoides pulcher* ex *Bufo japonicus*	LC018444	

(60%) were infested by only one parasite species, 21% by the trematode and one nematode species, while a simultaneous infection of two distinct nematode species was not observed. Trematode cysts were attached exclusively to the wall of the body cavity, mostly at the dorsal part. All adult nematodes were found within the digestive tract of the newt, whereas larval stages also moved through the body cavity. Lungs, liver, kidneys, bladder and reproductive system were found to be not infected.

Table 4. Helminth parasites of smooth newts *L. vulgaris* at two localities in the study area. Local infection rates of newts as well as developmental stages of the detected parasites are given.

Taxon	Locality 1	Locality 2
Parastrigea robusta	73% (1–23 metacercariae)	10% (7–8 metacercariae)
Megalobatrachonema terdentatum	29% (1–7 adults + larvae)	25% (1–4 adults + larvae)
Oswaldocruzia filiformis	6% (2–5 adults)	5% (2 adults)
Cosmocerca longicauda	2% (1 adult)	5 % (4 adults)
Unidentified cosmocercid nematode	2% (1 adult)	–
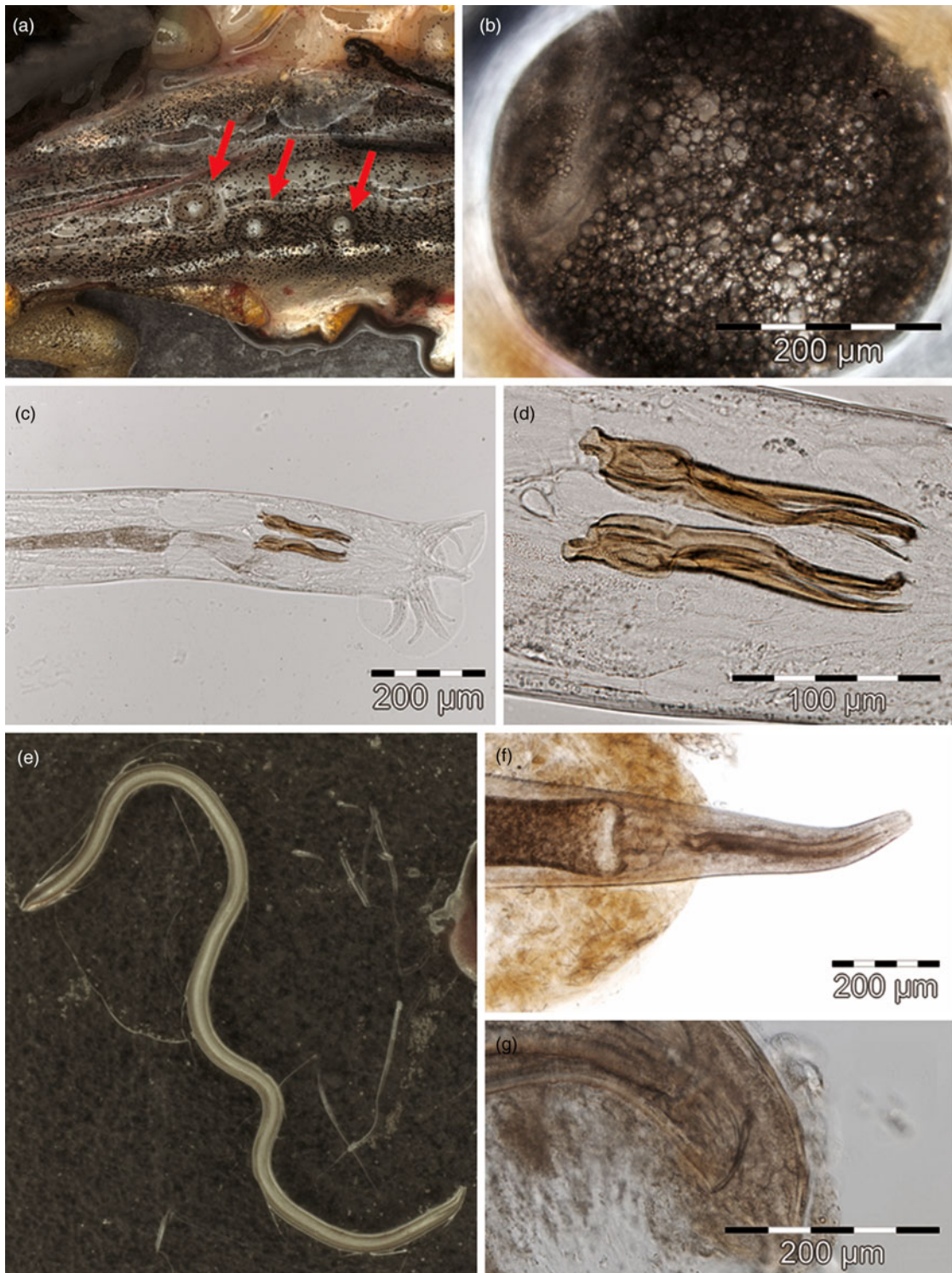
Fig. 1. Morphological features of smooth newt parasites. (a) Dorsal body cavity wall with three encysted metacercariae of *P. robusta* (indicated by red arrows), (b) metacercaria within the hyaline inner cyst wall, showing lipid globes within the paranephridial plexus. (c) Posterior extremity of a male *O. filiformis* (= *O. molgeta*) with spicules and bursa, (d) spicules. (e) Female *M. terdentatum* of length 22.7 mm. (f) Anterior extremity with oesophagus and bulbus of a male *C. longicauda*, (g) posterior extremity with speculum.

Encysted metacercariae, identified as Parastrigea robusta Szidat, 1928

Morphological diagnosis

The detected cysts (fig. 1a, b) showed the typical features of strigeid tetracotyle metacercariae, i.e. the inner hyaline cyst wall is of parasite origin, the outer walls of host origin, and reserve bladder forms network filling entire body, with free excretory bodies in canals (Dubois, 1968; Niewiadomska, 2002). Average life size of seven ellipsoid cysts under slight coverslip pressure was 356 µm (286–407) × 301 µm (241–336). They were compared to strigeid
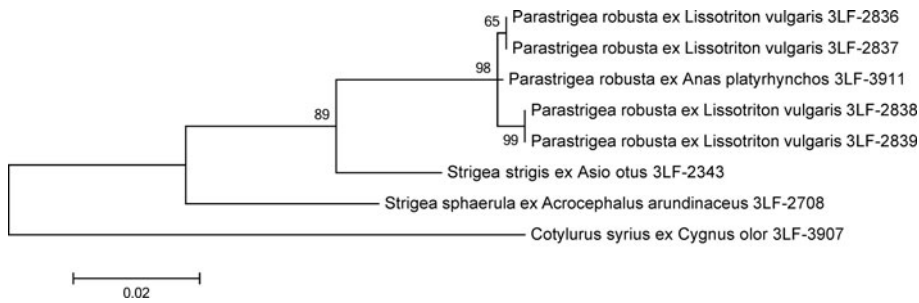
Fig. 2. Maximum likelihood analysis of concatenated nuclear and mitochondrial DNA loci from *P. robusta* and related species of Strigeidae. The scale bar indicates the number of substitutions per one nucleotide. Support values of maximum likelihood bootstrap analysis (1000 replicates) are indicated.

trematodes that are known to form metacercaria cysts in smooth newts or other central European amphibians, i.e. *P. robusta* (264–388 μm × 228–308 μm; Odening, 1965), *Strigea falconis* (440–607 μm × 220–404 μm; Odening, 1967), *S. sphaerula* (440–734 μm × 301–499 μm; Odening, 1967) and *S. strigis* (573–1049 μm × 360–433 μm; Odening, 1967). Consequently, the measured cyst size range matched exclusively with data on *P. robusta* in the literature, providing a tentative narrowing among candidates.

Molecular diagnosis

The DNA isolated from the cysts was variable in the CO1 locus (eight informative sites within the 368-bp-long sequence), whereas all the analysed nuclear loci (two partial 18S rDNA sequences, complete ITS1, 5.8S rDNA, ITS2 and partial 28S rDNA) were invariant. The 18S rDNA, ITS2 and CO1 sequences of an adult *P. robusta* obtained previously from its definitive host *Anas platyrhynchos* were identical with those of the cysts. ITS1, 5.8S rDNA and partial 28S rDNA were not available for the adult specimen. Based on the maximum likelihood analysis (fig. 2), DNA from all the four cysts was identified as *P. robusta*. The analysis of concatenated DNA sequences of ITS2, 18S rDNA and CO1 showed that the four sequences were 99.8–99.9% identical with the control sequence of an adult *P. robusta*. Conspecificity with *S. sphaerula* or *S. strigis* could therefore be excluded.

Nematode species 1, identified as Oswaldocruzia filiformis (Goeze, 1782)

Morphological diagnosis

The absence of a buccal ring and extradorsal rays indicated the taxonomic assignment of this male trichostrongylid nematode to the family Molineidae (Anderson *et al.*, 2009). Generic allocation to *Oswaldocruzia* was supported by the shape of the bursal dorsal ray, multiple processes of the spicules (fig. 1c, d) and the absence of a gubernaculum (Travassos, 1937). Morphological features of the specimen corresponded to those of *O. molgeta* (Lewis, 1928; Barus & Groschaft, 1962), but also fell into the range of

variability found in *O. duboisi*, *O. filiformis* and *O. goezei* (Moravec & Vojtková, 1975; Ben Slimane *et al.*, 1993). As morphological diagnostic features have not yet been proposed to distinguish among these taxa, assignment of the examined specimen to any taxon remained unreliable.

Molecular diagnosis

Using primers targeting partial 18S rDNA and ITS1–5.8S rDNA–ITS2 loci, DNA sequences for both loci in one specimen and for 18S rDNA in another were obtained (table 3). Specimen ZX3-1701 could be confirmed to be conspecific with *O. filiformis* obtained from *Pelophylax* [*Rana*] *ridibunda* by 99% (sequence similarity 99% at the ITS1 locus; 635/638 identities without gaps) (fig. 3). At the 18S rDNA locus, specimens examined showed a sequence similarity of 100% with *O. filiformis* (since the sequence of specimen ZX3-1701 has only a 437-nucleotide overlap with the respective *O. filiformis* database entry, this locus was not chosen for phylogeny) and of 90% with *Spiculopteragia houdemeri* (Trichostrongylidae: Ostertagiinae) as the closest match in GenBank (803/893 identities and 47 gaps in the alignment).

Nematode species 2, identified as Megalobatrachonema terdentatum (Linstow, 1890)

Morphological diagnosis

Adult specimens examined (fig. 1e) showed the typical morphological features of helminths of the genus *Megalobatrachonema* (Kathlaniidae) in having a cylindrical oesophageal isthmus with a glandular bulb lacking valves and poorly developed lips (Anderson *et al.*, 2009). The morphological features of these adult nematodes entirely matched those of *M. terdentatum* (Linstow, 1890) as described in Hartwich (1960) and Bertman & Okulewicz (1987). Larval specimens (stages III and IV) found in the body cavity lacked reliable diagnostic features (Petter & Chabaud, 1971).
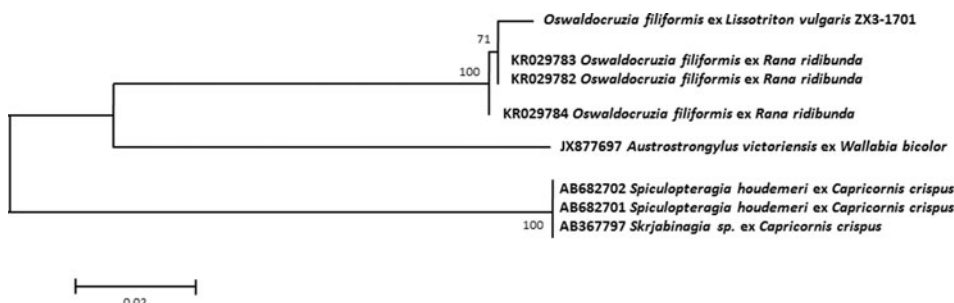
Fig. 3. Maximum likelihood analysis of ITS1–5.8S rDNA–ITS2 loci of an *O. molgeta* specimen isolated from the small intestine of the smooth newt *L. vulgaris* (ZX3-1701) and related nematode species. Support values of maximum likelihood bootstrap analysis (1000 replicates) are given. Scale bar = substitutions per site.

Fig. 4. Maximum likelihood analysis of concatenated partial 18S rDNA and ITS1 sequences of the nematodes isolated from the small intestine of the smooth newt *L. vulgaris* and related species. Support values of maximum likelihood bootstrap analysis (1000 replicates) are given. Scale bar = substitutions per site.
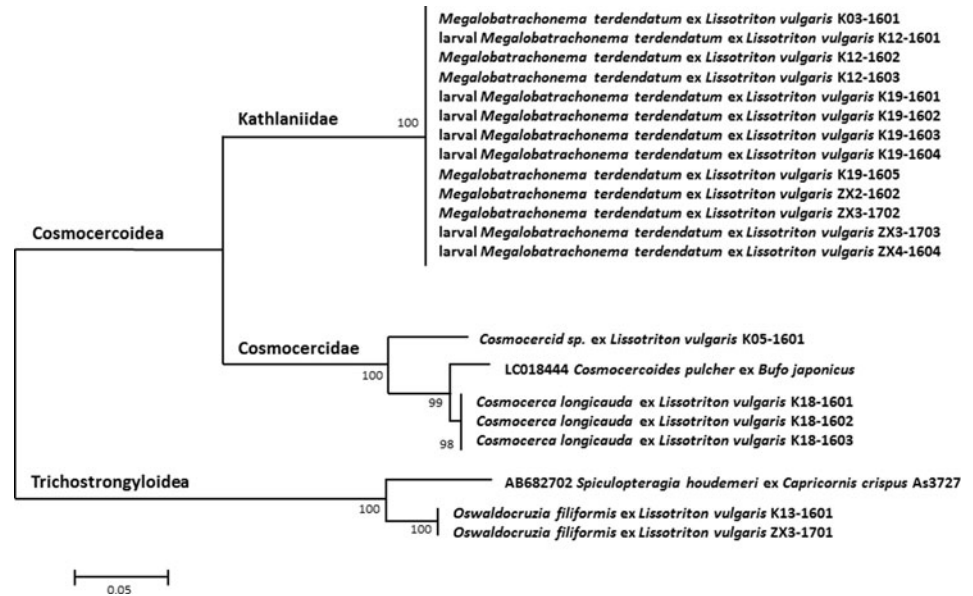
Molecular diagnosis

Using primers targeting 18S rDNA, sequences were obtained for a total of 13 specimens, including six morphologically diagnosed adults and seven undetermined larval stages collected moving in the body cavity (table 3). All the sequences obtained were identical. The closest match to sequences deposited in GenBank yielded a similarity of 94% with *Cosmocercoides pulcher* (Cosmocercidae: Cosmocercinae) (fig. 4).

Nematode species 3, identified as Cosmocerca longicauda (Linstow, 1885)

Morphological diagnosis

Four adult specimens of this nematode taxon resulted from a single newt individual. Three of them were inspected briefly before storing for molecular identification. The fourth specimen, examined morphologically, was *C. longicauda* (Cosmocercidae), exhibiting the features of a young male according to Grabda-Kazubska (1974) (fig. 1f, g).

Molecular diagnosis

Using primers targeting 18S rDNA, the sequences for the three specimens turned out to be identical (table 3). The closest match to sequences deposited in GenBank was *C. pulcher* (Cosmocercidae) showing a sequence similarity of 95% (fig. 4).

Unidentified nematode species 4

Morphological diagnosis

One nematode specimen, looking distinct from *M. terdentatum* and *C. longicauda*, was collected from the large intestine of a newt and identified briefly as a Cosmocercidae taxon before storing for molecular identification. To avoid any contamination of the sample, a thorough morphological examination of the specimen was avoided.

Molecular diagnosis

Using primers targeting 18S rDNA, a sequence for this individual specimen was obtained (table 3). The genetic similarity among this taxon and the identified Cosmocercoidea species *M. terdentatum* and *C. longicauda* was 70–72% and 85%, respectively. The closest match to sequences deposited in GenBank was again *C. pulcher* (Cosmocercidae: Cosmocercinae) with a similarity of 95% (fig. 4).

Discussion

Studying an amphibian's helminth community is a non-trivial taxonomic task because all morphological features of specimens collected should be linked to molecular data to confirm the identification, especially in areas where the avifauna is widespread and diverse (Patrelle *et al.*, 2015). This study provides the DNA sequences for five parasitic helminth species infesting smooth newts, from which four can be linked to unequivocally identified specimens. Therefore the number of newt parasites with a morphology–sequence link increased from six to seven trematode species (*P. robusta* added) and from three to five nematode species (*C. longicauda* and *M. terdentatum* added). Moreover, morphologically indistinct digenean metacercariae and larval nematode stages collected in surveys of naturally infected newts were linked with adults of *P. robusta* and of *M. terdentatum*. Our data contribute significantly to the building of a molecular library, for a better understanding of life cycles and host specificity of helminths infesting the widely distributed host, *L. vulgaris*.

Parastrigea robusta

Digenean cysts found in most newt individuals are difficult to associate with a distinct species because *P. robusta*, *S. sphaerula* and *S. strigis* are found to infest *L. vulgaris* in the wild, and *Holostephanus volgensis* in experimental trials (Vojtková, 1966; Vojtková & Vojtek, 1972; Shimalov *et al.*, 2001). As the morphological features of strigeid metacercaria cysts in amphibian hosts are not reliably distinctive at the species level (Nieawiadomska, 1970), they are often lumped together to a single larval genus *Tetracotyle* (e.g. Vojtková, 1963a, b; Vojtková & Roca, 1994). Morphometric features of the cysts seem to distinguish among potential taxa infesting *L. vulgaris* (Odening, 1965, 1967;

Vojtková, 1966), but variability within and among host species is not fully explored, leading to a considerable uncertainty. Using the molecular markers of morphologically identified adult parasites from the definitive host, the mallard (*A. platyrhynchos*), the metacercaria cysts of newts were confirmed as *P. robusta* cysts.

Commonly, the first intermediate host species of *P. robusta* in the wild are the planorbid snails *Anisus vortex*, *Gyraulus albus*, *Planorbis planorbis* and *Segmentina nitida* (Odening, 1965; Vojtek, 1989), none of which has been recorded until present in the breeding ponds of this surveyed newt population (Sinsch & Breuer, 2018). Ongoing molecular research will reveal whether or not the locally common *Gyraulus parvus* is a new first intermediate host species in the local *P. robusta* life cycle. Second intermediate host species in the wild are the newts *Triturus cristatus* and *L. vulgaris* (both present at the study site) and the brown frogs *Rana arvalis* and *R. temporaria* (Odening, 1965; Vojtková & Vojtek, 1972; Vojtek, 1989). Unlike *T. cristatus* in the Czech Republic, none of the crested newts reproducing in pond 1 was infested by *P. robusta* metacercariae (Sinsch, unpubl. obs. (2017) on ten individuals), whereas 73% of the smooth newts were infested. The observed infestation rate contrasts sharply with only 2.5% in *R. arvalis* (Zhigileva & Kirina, 2015) and the absence of *P. robusta* in the brown frogs of the Berlin and Cologne regions (Spieler, 1990; Andreas, 2007). Definitive hosts of *P. robusta* in the wild are *A. platyrhynchos*, *Aythya ferina*, *A. fuligula* and *Tachybaptus ruficollis* (Szidat, 1929; Bykhovskaya-Pavlovskaya, 1962; Dubois, 1968; Sitko, 1969; Macko, 1974; Storer, 2000; Sitko et al., 2006). The type host *A. platyrhynchos* is common in our study area, whereas other potential hosts are absent. In conclusion, the local life-cycle variant of *P. robusta* in the ponds surveyed includes a not yet evidenced planorbid snail, to the best of our knowledge probably *G. parvus*, exclusively smooth newts as the host of metacercariae and mallards as the definitive host.

Oswaldocruzia filiformis

Four species of *Oswaldocruzia* nematodes of doubtful validity have been reported from the intestines of *L. vulgaris*: *O. duboisi*, *O. filiformis*, *O. goezei* and *O. molgeta* (Vojtková, 1963a; Avery, 1971; Ben Slimane et al., 1993). Morphological variability of diagnostic features led to the proposal that the latter three are conspecific and should be referred to as *O. filiformis* (Moravec & Vojtková, 1975). Consequently, our study links the molecular identification of *O. filiformis* solely to morphologically identified *O. molgeta*, which is most probably a junior synonym of *O. filiformis*. We consider *O. duboisi* as erroneously assigned *O. filiformis* in a single *L. vulgaris* specimen of the collection of the Natural History Museum of Paris (Ben Slimane et al., 1993). *Oswaldocruzia goezei* reported from smooth newts in Poland (Taranko-Tulecka, 1959) and the Czech Republic (Vojtková, 1963a) was later assigned to *O. filiformis* (Moravec & Vojtková, 1975). In conclusion, *O. filiformis* remains the only confirmed and published *Oswaldocruzia* nematode infesting *L. vulgaris* as definitive host so far. The observed infestation rates of 5–6% in the studied newts are low compared with those of *L. vulgaris* in the Czech Republic (37.3%, Barus & Groschaft, 1962; 16.6–75.8%, Vojtková, 1963a) and in Greece (33%, Sattmann, 1990).

Megalobatrachonema terdentatum

Despite being a commonly observed nematode in the intestine of many European amphibian species (Vojtková, 1963a; Vojtková &

Roca, 1996), this is the first study to provide DNA sequences for *M. terdentatum*. Molecular markers proved to be particularly useful to identify the morphologically indistinct larval stages found moving through the body cavity of several of the newts examined (Petter & Chabaud, 1971). The observed infestation rates of 25–29% in the studied newts are in agreement with those in *L. vulgaris* in the Czech Republic (16.6–31%, Vojtková, 1963a).

Cosmocercid nematode species

Two distinct cosmocercid nematode species were found in the intestine of one and two newts, respectively. As the more abundant species was morphologically identified as *C. longicauda*, our molecular data deposited in GenBank represent the first link of a DNA sequence with this nematode species. In contrast, the other nematode sequence cannot be assigned to a species because the only specimen detected was not examined morphologically for species identification. Given that the specimen does not belong to a not yet recorded parasite of *L. vulgaris*, possible species identities are *C. ornata* (Shimalov et al., 2001) or *O. brevicaudatum* (e.g. Barus & Groschaft, 1962). The maximum likelihood tree (fig. 4) does not indicate a close relationship between *C. longicauda* and the unidentified nematode, so that it appears more likely to be *O. brevicaudatum* than *C. ornata*. Further collection of specimens will help to overcome this unresolved identity. The observed infestation rates (*C. longicauda*) of 2–5% in the studied newts are considerably lower compared to those of *L. vulgaris* in the Czech Republic (34.4%, Barus & Groschaft, 1962). If the unidentified nematode was *O. brevicaudatum*, the observed infestation would be comparable to that found in the Czech Republic (2.2%, Barus & Groschaft, 1962).

In conclusion, the parasitic helminth diversity using smooth newts as intermediate or definitive hosts is lower than previously perceived (28 instead of 31 species), including two Acanthocephala and Cestoda each, 14 Trematoda and ten Nematoda species.

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