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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

Omar M. Amin[1], **Richard A. Heckmann**[2], **Žiga Fišer**[3], **Valerija Zakšek**[3], **Holger Herlyn**[4], **Rok Kostanjšek**[3]

[1] Institute of Parasitic Diseases, Scottsdale, Arizona, USA;

[2] Department of Biology, Brigham Young University, Provo, Utah;

[3] Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia;

[4] Institute of Organismic and Molecular Evolution, Anthropology, Johannes Gutenberg University, Mainz, Germany.

Abstract: *Acanthocephalus balkanicus* Batchvarov et Combes, 1974 was incompletely described from the northern crested newt, *Triturus cristatus* (Laurenti) (Amphibia: Salamandridae), a possible synonym of the Balkan crested newt, *Triturus ivanbureschi* Arntzen et Wielstra, from a pond in village of Pesnopoy, southern Bulgaria. We provide a full description of adult males and females of the same taxon from the olm, *Proteus anguinus* Laurenti (Amphibia: Proteidae), the only exclusively aquatic cave-dwelling vertebrate in Europe, captured in Postojna-Planina Cave System in Slovenia. Cystacanths were also collected from the cave ecomorph of *Asellus aquaticus* (Linnaeus) (Crustacea: Asellidae) in the same location. Molecular analysis of specimens from Slovenia revealed that they are genetically almost identical to those of *Acanthocephalus anguillae* (Müller, 1780), a common parasite of European freshwater fishes. We propose to recognise the morphological and host differences by describing *A. balkanicus* as a new subspecies of *A. anguillae*. *Acanthocephalus anguillae balkanicus* is rather small and cylindrical with cylindrical proboscis having 10 rows of 6 hooks with simple roots each, long neck, large balloon-shaped lemnisci, small spherical anterior testis, and 6 club-shaped cement glands in 3 pairs. SEM images reveal more morphological details and the X-ray scans of gallium cut hooks shows considerably higher levels of phosphorus and calcium in adult hooks than in cystacanth hooks, especially in basal areas. Sulfur levels were higher in the arch and basal area of cystacanth hooks than adult hooks. Considering that both definitive and intermediate hosts of the Slovenian population of this acanthocephalan are bound to cave life, it is possible that its entire life cycle is uniquely completed underground.

Keywords: new subspecies, acanthocephalans, olm, EDXA analysis, molecular profile, Postojna-Planina Cave System.

Amin (2013) recognised 53 species of the acanthocephalan genus *Acanthocephalus* Koelreuther, 1771 mostly from freshwater fishes and amphibians. With the description of *Acanthocephalus rhinensis* Amin, Thielen, Münderle, Taraschewski et Sures, 2008 from the European eel, *Anguilla anguilla* (Linnaeus) in Germany, Amin et al. (2008) documented 10 species of *Acanthocephalus* in Europe, 6 from fishes and 4 from amphibians. Acanthocephalans recently collected from the olm, *Proteus anguinus* Laurenti (Amphibia: Proteidae), and from the cave ecomorph of *Asellus aquaticus* (Linnaeus) (Crustacea: Asellidae) in Postojna-Planina Cave System in Slovenia (Fig. 1) were readily recognisable as *Acanthocephalus balkanicus* Batchvarov et Combes, 1974 using the key in Amin et al. (2008).

However, the original description of *A. balkanicus* from the northern crested newt, *Triturus cristatus* (Laurenti) (a possible synonym of the Balkan crested newt. *Triturus ivanbureschi* Arntzen et Wielstra) (Amphibia: Salamandridae), from a pond in southern Bulgaria (Fig. 1) was largely incomplete and lacking in important measurements and illustrations (Batchvarov and Combes 1974). Furthermore, type specimens of the original species description were not available for reinvestigation. Our recent collections from Slovenia provided an opportunity to fully describe the species and fill in the many gaps in its original description. As molecular analysis revealed that specimens collected in Slovenia are genetically almost identical to those of *Acanthocephalus anguillae* (Müller, 1780), we herein propose and describe *A. balkanicus* as a subspecies of *A. anguillae.*

Address for correspondence: Omar M. Amin, Institute of Parasitic Diseases, 11445 E. Via Linda 2-419, Scottsdale, Arizona 85259, USA. E-mail: omaramin@aol.com

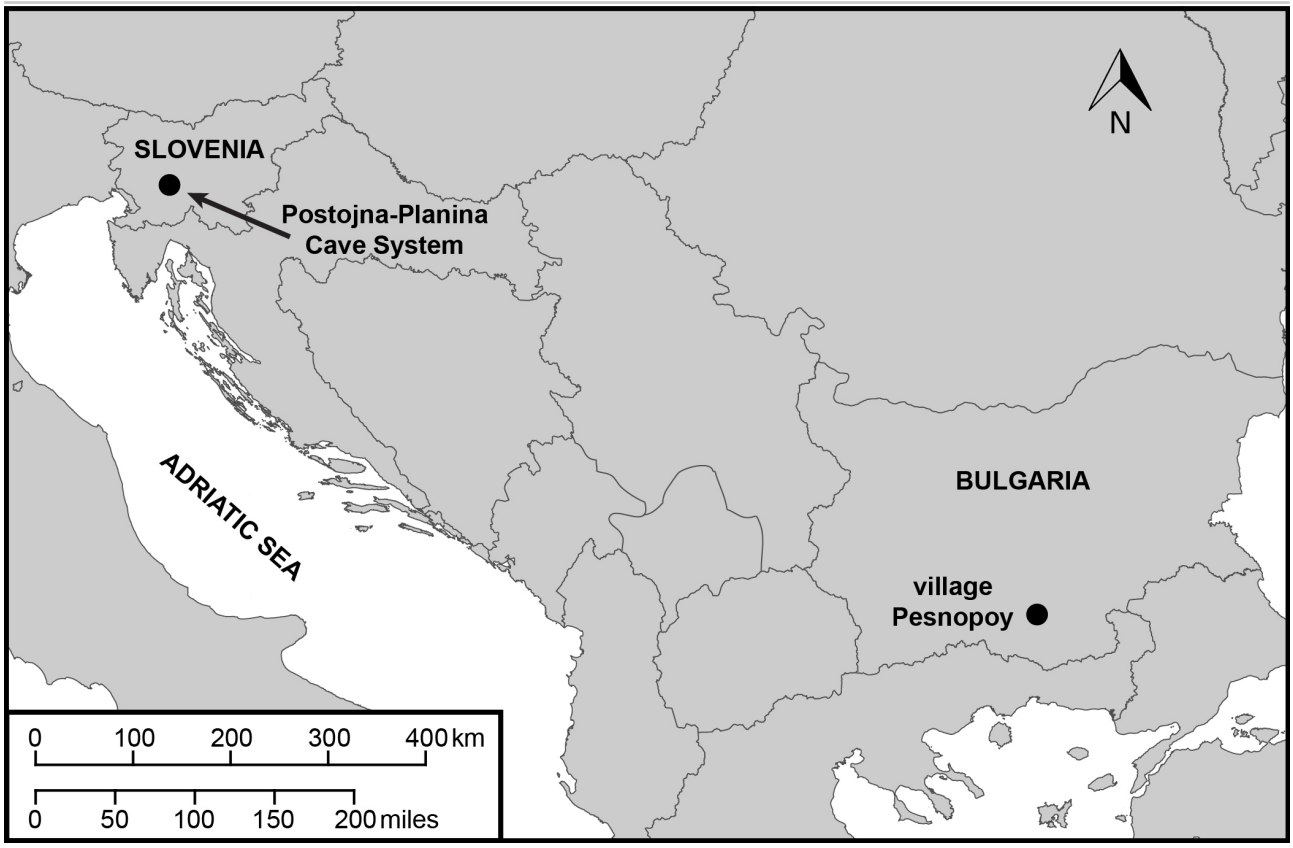


Fig. 1. Map of collecting sites of *Acanthocephalus anguillae balkanicus* subsp. n. in Slovenia and Bulgaria.

MATERIALS AND METHODS

Characteristics of locality and hosts

Specimens of *Acanthocephalus anguillae balkanicus* subsp. n. were collected from the olm, *Proteus anguinus* (definitive host), and from the cave ecomorph of waterlouse, *Asellus aquaticus* (intermediate host), in the Postojna-Planina Cave System (PPCS; WGS 84 coordinates at the cave entrance: 45°49'12"N; 14°14'45"E) in Slovenia (Fig. 1). This complex cave system comprises more than 30 km of subterranean habitat. Specimens used in this study were collected in different yet connected parts of the PPCS (see below and consult Gams 2004 and Šebela 2012 for more information regarding the distinct parts of this cave system).

The Postojna-Planina Cave System likely harbours the richest obligate cave-dwelling fauna in the world, with almost 100 such species (Sket 2012), including a large population of olms (Zakšek et al. 2018), the only obligate cave-dwelling vertebrate species in Europe. This paedomorphic salamander remains aquatic throughout its life; it eats, sleeps, and breeds underwater. It is highly adapted to life in complete darkness (Gorički et al. 2012). The olm feeds on crustaceans, snails, and insects (Aljančič et al. 1993). Among its crustacean prey are mostly cave shrimps *Troglocaris* Dormitzer and cave amphipods *Niphargus* Schiödte, but also the waterlouse, *A. aquaticus*. The latter is a detritivore generalist, common in most European surface freshwaters, but inhabits also several subterranean habitats including waters in the PPCS (Sket 1994). The cave populations therein are morphologically and genetically distinct from the nearby surface populations (Verovnik et al. 2003, 2004; Prevorčnik et al. 2004; Konec et al. 2015), and their individuals are referred to as the cave ecomorph of *A. aquaticus* in this paper.

Study specimens

For morphological analyses all specimens used were collected in Pivka Channel of Planina Cave (Slovene name: Pivkin rokav Planinske jame), a part of PPCS. One, approx. 20, and 2 adult parasites were collected from three different olms in October 2008, July 2014, and February 2016, respectively. Cystacanths were collected from the cave ecomorph of *A. aquaticus* in June, 2014. Approximately 150 isopods were examined and the infection rate was about 40%. Almost exclusively, isopods carried a single cystacanth. Parasites sent to the Scottsdale laboratory were processed as described below.

For molecular analyses we used an adult parasite (voucher no. BA053) collected from a single olm from Črna jama (Vilharjev rov), a part of PPCS, on 26 April 2018, and a cystacanth (voucher no. BA020) collected from the cave ecomorph of *A. aquaticus* in Pivka jama, a part of the PPCS, on 27 June 2015. Additionally, we used two *A. anguillae* specimens (voucher nos. BA051, BA052) obtained in 2014 from the European eel, *Anguilla anguilla*, from the river Weser at Gieselwerder close to Kassel, Hesse, Germany.

All acanthocephalan specimens obtained from the olms were collected under permits (nos. 35701-81/2004-9, 35601-1/2010-6, 35601-8/2016-4) issued by the Slovenian Ministry of Environment and Spatial Planning. Additional parasites from both cave-dwelling hosts are kept in the Zoological Collection at the Department of Biology, Biotechnical Faculty, University of Ljubljana. The eel from river Weser was caught and sacrificed by an authorised person.

Procedures for light microscopy

Worms processed for microscopical examination were punctured with a fine needle and subsequently stained in Mayer's acid

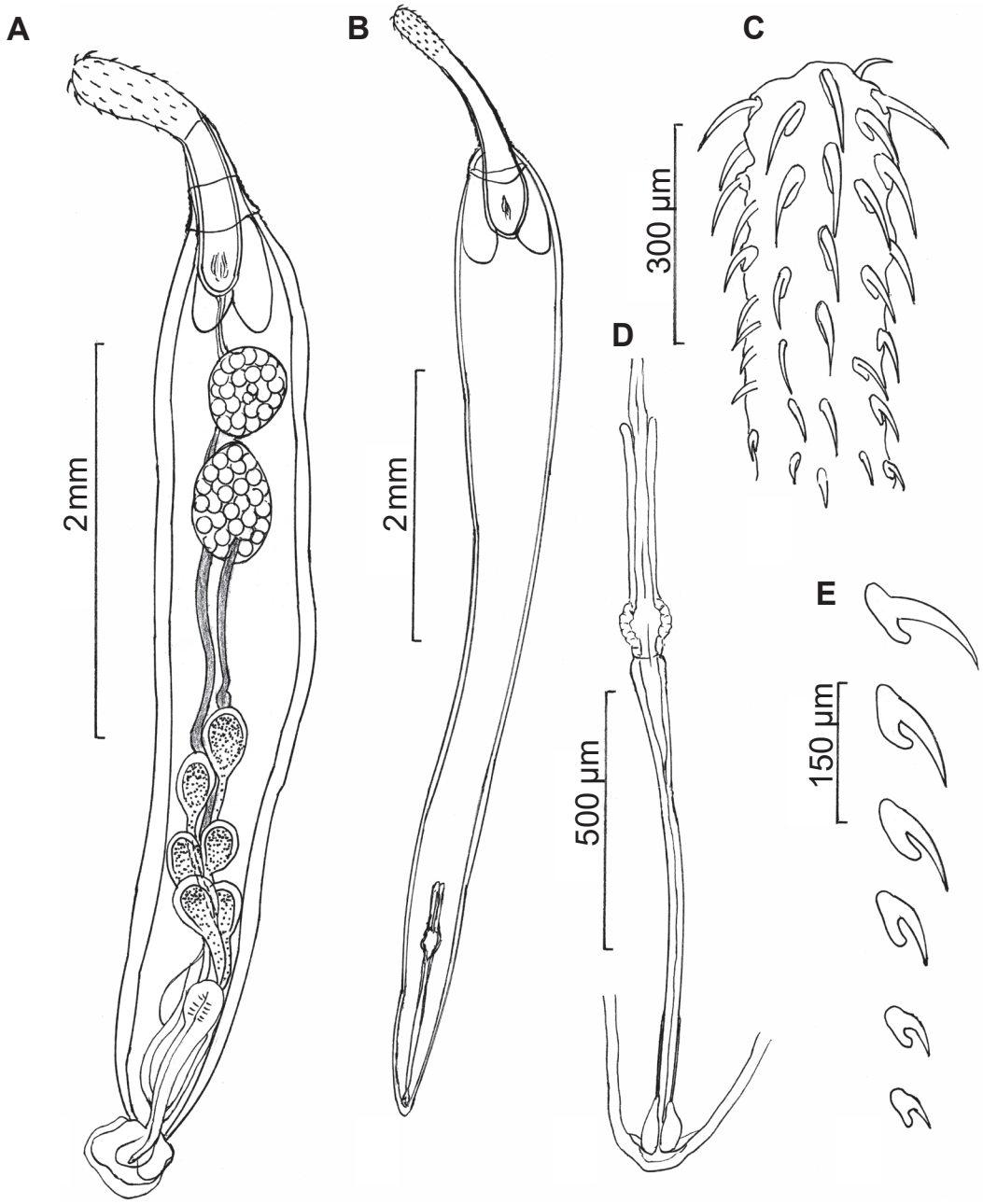


Fig. 2. Line drawings of *Acanthocephalus anguillae balkanicus* subsp. n. from *Proteus anguinus* Laurenti. **A** – male holotype; **B** – female allotype; **C** – the proboscis of a male specimen; ventral hooks on right; **D** – the reproductive system of a female paratype; note the simple vagina, long slender uterus, the spherical muscular base of the long undifferentiated uterine bell, and the absence of uterine bell glands; **E** – a row of ventral hooks from the male specimen in Fig. 2C; note the progressively decreasing size of hook roots and anterior manubria posteriorly.

carmine, destained in 4% hydrochloric acid in 70% ethanol, dehydrated in ascending concentrations of ethanol (24 hr each), cleared in 100% xylene and then transferred into 50% Canada balsam and 50% xylene (24 hr each). Whole worms were then mounted in Canada balsam. All measurements are in micrometers, unless otherwise noted; the range is followed by the mean value between parentheses. Width measurements represent maxima. Trunk length does not include proboscis, neck or bursa. Line drawings were created using a Ken-A-Vision micro-projector (Ward's Biological Supply Co., Rochester, New York, USA) which uses cool quartz iodine 150 W illumination. Colour-coded objective (10×, 20×, 43×) lenses were used. Images of stained whole mounted specimens were projected vertically on 300 series Bristol draft paper (Starthmore, Westfield, Massachusetts, USA), then traced and inked with India ink. Projected images were identical to the actual specimens being projected. Type specimens were deposited at the University of Nebraska's State Museum's Harold W. Manter Laboratory (HWML) collection in Lincoln, Nebraska, USA.

Scanning electron microscopy (SEM)

Samples of parasites that had been fixed and stored in 70% ethanol were processed following standard methods. These included critical point drying (CPD) in sample baskets and mounting on SEM sample mounts (stubs) using conductive double sid-

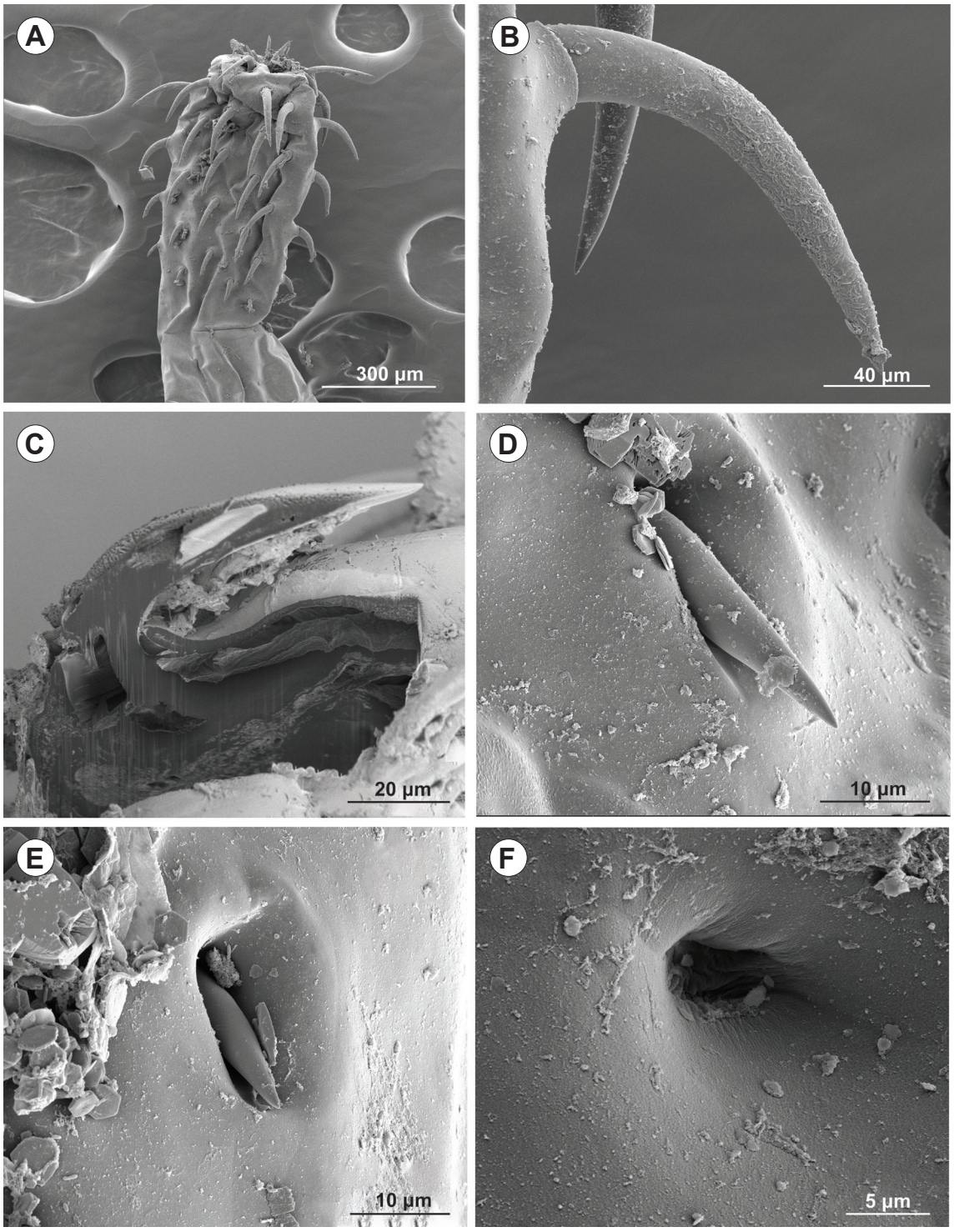


Fig 3. SEM images of *Acanthocephalus anguillae balkanicus* subsp. n. specimens from *Proteus anguinus* Laurenti. **A** – proboscis of a paratype male specimen; **B** – the anterior hook on the proboscis shown in Fig. 3A; **C** – a gallium-cut anterior hook showing its solid core; **D** – a hook at the middle of the proboscis; **E** – a posterior hook; **F** – a sensory pore in the neck.

ed carbon tape. Samples were coated with gold and palladium for 3 minutes using a Polaron #3500 sputter coater (Q150T ES, Quorum Technologies, UK) establishing an approximate thickness of 20 nm. Samples were placed and observed in a JSM-7500F Field Emission Scanning Electron Microscope (JEOL, Tokyo, Japan) or a Helios Nanolab 600 DualBeam Scanning Electron Microscope and digital images were obtained using the Nanolab software system (FEI, Hillsboro, Oregon). Images were taken at various magnifications under low vacuum conditions of 93 Pa using 10 kV, spot size 2 and a GSE detector in a Helios Nanolab 600 microscope, and under high vacuum conditions of 5×10^{-5} Pa and 2 kV in a JSM-7500 microscope.

Energy dispersive analysis for X-ray (EDAX)

Standard methods similar to the above SEM procedure were used for specimen preparation. Specimens were examined and

Table 1. A list of taxa, samples, and corresponding GenBank accession numbers of sequences used in phylogenetic analysis.

Taxon	Host	Location	Voucher	COI	18S rRNA	ITS	28S rRNA	Reference
Acanthocephalus anguillae balkanicus	*Proteus anguinus*	Črna jama (Vilharjev rov), PPCS, Postojna, Slovenia	BA053	MN416029	MN394414	MN394424	MN394419	this study
Acanthocephalus anguillae balkanicus	*Asellus aquaticus* (cave ecomorph)	Pivka jama, PPCS, Postojna, Slovenia	BA020	MN416030	MN394415	MN394426	MN394421	this study
Acanthocephalus anguillae	*Anguilla anguilla*	river Weser at Giedelwerder, Kassel, Hesse, Germany	BA051	MN416027	MN394412	MN394422	MN394417	this study
Acanthocephalus anguillae	*Anguilla anguilla*	river Weser at Giedelwerder, Kassel, Hesse, Germany	BA052	MN416028	MN394413	MN394423	MN394418	this study
*Acanthocephalus dirus**	*Asellus aquaticus* (surface ecomorph)	unknown	/	DQ089718	AY830151	/	AY829106	García-Varela and Nadler (2005, 2006)
Acanthocephalus anguillae	*Asellus aquaticus* (surface ecomorph)	Ouche River, Dijon, France	/	/	LS991432	/	/	Perrot-Minnot (unpublished)
Acanthocephalus anguillae	*Barbus barbus*	river Weser, near Göttingen, Germany	/	/	AF469413	/	/	Herlyn et al. (2003)
Acanthocephalus ranae	*Asellus aquaticus* (surface ecomorph)	Ouche River, Dijon, France	/	/	LS991433	/	/	Perrot-Minnot (unpublished)
Acanthocephalus lucii	*Perca fluviatilis*	Nottinghamshire, Bleasby Lake, United Kingdom (18S); unknown (28S)	/	/	AY830152	/	KM656148	García-Varela and Nadler (2005); Wayland et al. (2015)
Acanthocephalus sp.	*Perca fluviatilis*	Geneva Lake, France	/	/	DQ147605	/	/	Nicoulaud et al. (unpublished)
Acanthocephalus nanus	*Cynops pyrrhogaster*	Japan	/	LC100070	LC129889	LC100043	LC100043	Nakao (2016)
Filisoma bucerium (outgroup)	*Kyphosus elegans*	unknown	/	/	AF064814	/	AY829110	García-Varela and Nadler (2005)

*According to our phylogenetic analysis this specimen is most probably *Acanthocephalus anguillae* and not *A. dirus*.

positioned with the before mentioned SEM instrument which was equipped with a Phoenix energy-dispersive X-ray analyzer (FEI, Hillsboro, Oregon). X-ray spot analysis and live scan analysis were performed at 16 kV with a spot size of 5. Results were recorded on charts and stored with Texture and Elemental Analytical Microscopy (TEAM) digital imaging software (FEI). The data included weight percent and atom percent of the detected elements following correction factors.

Ion sectioning of hooks

A dual-beam SEM with a gallium ion source (GIS) was used for the liquid metal ion source (LMIS) part of the process. The hooks of acanthocephalans were sectioned using a probe current between 0.2 nÅ and 2.1 nÅ according to the rate at which the area was cut. The time of cutting depended on the nature and sensitivity of the tissue. Following the initial cut, the sample was milled to obtain a smooth surface. The cut was then analyzed for ions with an electron beam (tungsten) to obtain an X-ray spectrum. The intensity of the GIS was variable due to the nature of the material being cut.

Procedures for molecular study

Genomic DNA was isolated from whole animals (excluding proboscis) using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) following the protocol for Mammalian Tissue Preparation. We amplified four genetic markers, one mitochondrial: a fragment of the gene for cytochrome oxidase I (COI), and three nuclear: gene for 18S rRNA, the internal transcribed spacer region (ITS1, 5.8 rRNA, ITS2), and a fragment of gene for 28S rRNA. The 660 bp long fragment of COI was amplified using primers LCO 1490 and HCO 2198 (Folmer et al. 1994), the approx. 1,700 bp long 18S rRNA gene was amplified using the forward primer 18S ACF (5′-AGATTAAGCCATGCATGCGTAAG-3′) and the reverse primer 18S ACR (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Verweyen et al. 2011), the approx. 1,500 bp long ITS was amplified using the forward primer ac58f (5′-GTCGTAACAAGGTTTCCGT-3′) and the reverse primer ac1500r1 (5′-CGATTGATTTGCACGTC-3′) (Tkach et al. 2013), and the approx. 1,500 bp long fragment of 28S rRNA gene was amplified using the forward primer 28S LSU6-3 (5′-GGAACCCTTCTCCACTTCAGTC-3′) and the reverse primer 28S LSU5 (5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′) (Littlewood et al. 2000).

PCRs were performed using the following cycling settings: initial denaturation at 94 °C for 3 min, followed by 60 s at 94 °C, 60 s at 46 °C, 120 s at 72 °C for 40 cycles, and final extension at 72 °C for 5 min for COI gene; initial denaturation at 94°C for 3 min, followed by 45 s at 94 °C, 60 s at 53 °C, 120 s at 72 °C for 40 cycles, and final extension at 72 °C for 5 min for 18S rRNA gene; initial denaturation at 94 °C for 3 min, followed by 45 s at 94 °C, 60 s at 48 °C, 150 s at 72 °C for 30 cycles, and final extension at 72 °C for 5 min for ITS and 28S rRNA gene. PCR products were purified using Exonuclease I and FastAP (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. All markers were sequenced in both directions using their respective PCR amplification primers. To get a complete overlap, ITS was sequenced with two additional internal primers (ac900F and ac900R; see Tkach et al. 2013 for primer sequences). Sequencing was performed by Macrogen Europe (Amsterdam, Netherlands). Chromatograms were assembled and edited using Geneious 11.0.3 (Biomatters, New Zealand).

All new sequences were deposited in GenBank under accession numbers provided in Table 1. Additionally, available and appropriate sequences of other *Acanthocephalus* species and specimens were retrieved from GenBank and included in phylogenetic analysis; *Filisoma bucerium* Van Cleave, 1940 was used as an outgroup representative (see Table 1 for details).

Sequences were aligned separately for each marker using MAFFT v.7 (Katoh and Standley 2013) and then concatenated into a final dataset with the total length of 4,445 bp. The overlapping region between ITS and 28S rRNA gene sequences was used only once. The best partitioning scheme and optimal substitution

models for the COI gene codon positions were searched for using PartitionFinder 2.1.1 (Lanfear et al. 2012). Phylogenetic relationships were reconstructed using Bayesian inference in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003). Bayesian MCMC tree search with two independent runs with four chains each was run for 10 million generations and trees were sampled every 1000 generations. After having validated that the stationary phase was reached, the first 25% of trees were discarded, and a 50% majority rule consensus tree was calculated from the remaining trees. Additionally, uncorrected genetic distances between specimens based on COI and 18S genes were calculated in MEGA X (Kumar et al. 2018).

RESULTS

Specimens collected from the olm *Proteus anguinus* and the cave ecomorph of *Asellus aquaticus* in Slovenia were recognisable as *Acanthocephalus balkanicus* as described by Batchvarov and Combes (1974), and were distinguishable from *Acanthocephalus anguillae* in morphology and definitive host. However, based on molecular evidence (see "Molecular findings" below), we propose *A. balkanicus* as a synonym of *A. anguillae*. We also reduce the status of *A. balkanicus* sensu stricto to a subspecies of *A. anguillae* sensu lato, as diagnosed below based on morphological, host and geographical distinctiveness. First, we provide an emended diagnosis of *A. anguillae* based on specimens collected from freshwater fishes in open waters throughout Europe as reported by Lühe (1911), Meyer (1932), and Petrochenko (1956), among others, as well as on specimens collected from caudate amphibians in a pond in Bulgaria and a cave in Slovenia as reported by Batchvarov and Combes (1974) and us in this paper, respectively. Second, we provide a description of *A. balkanicus* sensu stricto as a new subspecies of *A. anguillae*, i.e., *Acanthocephalus anguillae balkanicus*, based on our examination of new material from Slovenia.

Diagnosis of *Acanthocephalus anguillae* sensu lato

With characters of the genus *Acanthocephalus*. Worms small to medium with cylindrical trunk slightly dilated anteriorly, reaching 9.5 × 1.1 mm and 30.0 × 2.2 in males and females, respectively. Proboscis somewhat club-shaped, slightly wider anteriorly, 580–1,000 long × 310–390 wide armed with 10 rows of 5–7 hooks each. Hooks longest anteriorly, well-developed, with vertical roots with prominent, absent or barely discernible oblique anterior lateral processes. Longest anterior most hooks (120 × 16 in males, 136 long in females) and roots (71 × 22 in males, 74 long in females); hooks nos. 3 and 4 from anterior most robust. Posterior most hook spiniform with diminished or reduced root, reaching 64 in length. Neck 400–700 × 230 wide posteriorly. Proboscis receptacle double walled, 1.08–1.70 × 0.17 mm, with cephalic ganglion at its base. Lemnisci sacciform, extending slightly posterior to receptacle, 1.0–1.70 × 0.23 mm. Testes oblong, equatorial or post-equatorial, contiguous, 1.35 × 0.47 mm or smaller pre-equatorial, contiguous, round, 0.36–0.55 × 0.28–0.41. Cement glands 6, round, in 3 pairs. Female reproductive system long, cylindrical, with terminal gonopore. Eggs fusiform with polar prolongation of fertilisation membrane, 68–110 × 10–12. In many species of freshwater fishes in open European waters extending east to the Ob River and the Far East (Petrochenko 1956) or in caudate amphibians in ponds in Bulgaria and caves in Slovenia.

The subspecies *Acanthocephalus anguillae anguillae*

With characters of *A. anguillae*. Medium size worms reaching 9.10–9.5 × 1.0–1.1 mm and 12.0–30.0 × 2.0–2.2 mm in males and females, respectively. No detectable dorso-ventral differentiation of proboscis hooks. Hook roots vertical with prominent oblique anterior lateral processes, except posterior most hooks with rudimentary root. Testes large, oblong, equatorial or post-equatorial. In many species of freshwater fishes in open European waters extending east to the Ob River and the Far East (Petrochenko 1956). Records from North America and Mediterranean are apparently in error; see Golvan (1969).

The subspecies *Acanthocephalus anguillae balkanicus* subsp. n.

With characters of *A. anguillae.* Small worms, trunk 2.40–4.30 × 0.60–0.70 mm and 3.50–7.10 × 0.50–0.90 mm in males and females, respectively (Fig. 2A,B), with all other characters correspondingly smaller. Proboscis hooks larger ventrally than dorsally (Fig. 2C). All hook roots prominent, manubriated anteriorly, vertical with absent or barely discernible oblique anterior lateral processes, smaller than blades (Fig. 2C,E). Testes small, round, pre-equatorial (Fig. 2A). In northern crested newts from ponds in Bulgaria and olms from caves in Slovenia.

Morphological description of adult *Acanthocephalus anguillae balkanicus* from *Proteus anguinus*

General: With characters of the genus *Acanthocephalus* (Echinorhynchidae). Trunk aspinose, small, cylindrical, gradually tapering at both ends. Body wall with micropores distributed somewhat evenly (Fig. 4C). Females slightly wider anteriorly (Figs. 2B, 4A). Shared structures relatively larger in females than in males. Body wall of same moderate thickness dorsally and ventrally (Fig. 2A). Proboscis cylindrical with 10 rows of 6 slender rooted hooks each (Figs. 2C, 3A). Apical hooks largest, gradually decreasing in size posteriorly (Fig. 3B–F). Ventral hooks longer and more robust than dorsal hooks (Fig. 2C). All hook roots prominent but shorter than blades, simple, directed posteriorly in sharp angle, with marked anterior manubria decreasing in size posteriorly (Fig. 2E). Neck prominent, almost as long as proboscis, wider posteriorly, in 2 parts; a slender anterior part with paired sensory pores (Fig. 3F) and corrugated more conical posterior part appearing as continuation of body wall (Figs. 2A,B, 4B). Proboscis receptacle double-walled, plump, about as long as proboscis, with cephalic ganglion at its base. Lemnisci rounded, equal, extending posterior to receptacle (Fig. 2A,B).

Male (based on 4 adults). Trunk 2.47–4.37 (2.18) mm long by 0.62–0.72 (0.67) mm wide. Proboscis 624–728 (673) long by 156–239 (210) wide. Length of dorsal hooks from anterior 122–125 (123), 80–112 (98), 80–112 (97),

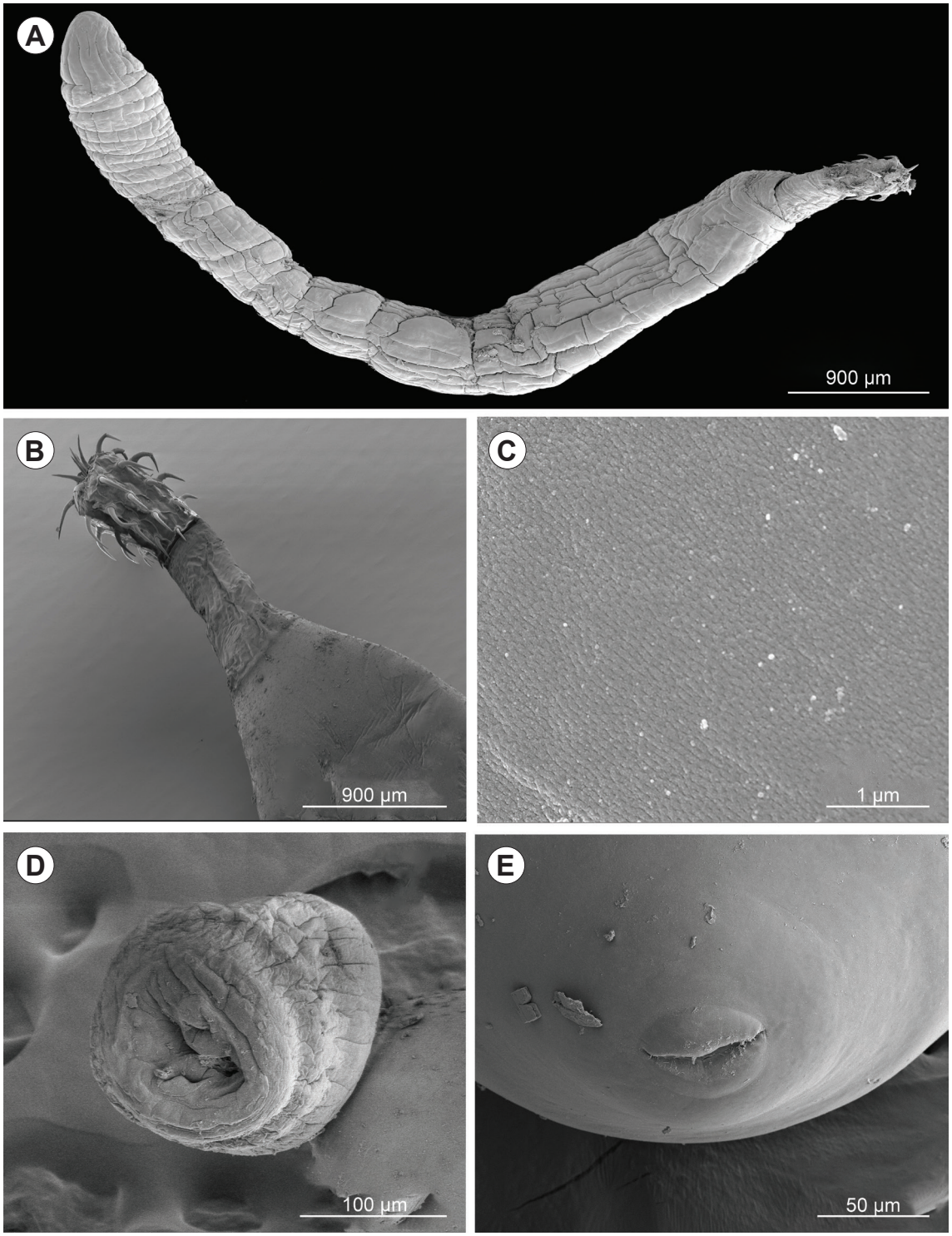


Fig. 4. SEM images of *Acanthocephalus anguillae balkanicus* subsp. n. specimens from *Proteus anguinus* Laurenti. **A** – a whole female specimen; **B** – the anterior portion of another specimen showing the shape of the proboscis, the elongated and basally enlarged neck, and the widening anterior trunk; **C** – micropores on the mid-trunk; **D** – a thick muscular bursa; note the lack of prominent sensory structures; **E** – the terminal gonopore of a female.

62–80 (74), 55–75 (63), 42–50 (46). Length of ventral hooks from anterior 130–138 (134), 117–125 (120), 90–125 (107), 77–114 (94), 72–87 (78), 50–63 (56). Anterior neck 333–364 (348) long by 156–218 (187) wide posteriorly. Posterior neck 260–291 (275) long by 291–333 (312) wide at junction with trunk. Receptacle 728–1000 (827) long by 175–208 (192) wide. Lemnisci 572 long by 125–260 (192) wide. Testes pre-equatorial near receptacle, round-ovoid, equal, and contiguous (Fig. 2A). Anterior testis 395–478 (444) long by 312–395 (348) wide. Posterior testis 364–551 (452) long by 281–416 (346) wide. Two prominent sperm ducts extending side by side between testes and cement glands. Six rounded-ovoid cement glands slightly larger anteriorly 156–312 (255) long by 146–250

(192) in 3 pairs each with its own duct. Cement gland ducts joining at base of Seafftigen's pouch, 312–468 (381) long by 166–256 (159) wide (Fig. 2A). Bursa muscular, with prominent rim but no apparent sensory structures (Fig. 4D), 281–312 (296) long by 291–312 (201) wide.

Female (based on 7 adults). Trunk 3.50–7.15 (4.72) mm long by 0.50–0.95 (0.76) mm wide anteriorly. Proboscis 676–988 (780) long by 208–260 (234) wide. Length of dorsal hooks from anterior: 175, 157–167 (162), 95–162 (145), 80–130 (99), 65–95 (75), 35–37 (36). Length of ventral hooks from anterior: 187–200 (193), 145–165 (153), 130–165 (143), 95–130 (110), 55–120 (84), 40–50 (45). Anterior neck 260–468 (343) long by 156–260 (231) wide posteriorly. Posterior neck 208–364 (291) long by 222–468 (337) wide at base. Proboscis receptacle 830–1040 (951) long by 218–302 (245) wide. Lemnisci 520–676 (607) long by 156–250 (212) wide. Reproductive system slender with terminal gonopore (Fig. 4E). Simple vagina without muscular sphincter and long uterus gradually enlarging into muscular rounded base of uterine bell (Fig. 2D), 1.40 mm long in 1 specimen. Eggs not available, only ovarian balls.

Description of cystacanths from the cave ecomorph of *Asellus aquaticus*

The general morphology of the trunk, proboscis and hooks, receptacle, and lemnisci of the cystacanths were comparable to that of the adults. Only the reproductive system appears underdeveloped.

Systematic summary

Type host: The northern crested newt, *Triturus cristatus* (Laurenti) (a possible synonym of the Balkan crested newt, *Triturus ivanbureschi* Arntzen et Wielstra) (Amphibia: Salamandridae).

New definitive host: Olm, *Proteus anguinus* Laurenti (Amphibia: Proteidae).

Intermediate host: Cave ecomorph of *Asellus aquaticus* (Linnaeus) (Crustacea: Asellidae).

Type locality: a pond near village Pesnopoy (WGS 84: 41°41'47"N; 25°7'31"E), Plovdiv, Bulgaria, Europe.

Locality: Pivka Channel of Planina Cave (WGS 84: 45°49'12"N; 14°14'45"E), Planina, Slovenia, Europe.

Specimens deposited: HWML 139428 (males and females on 1 slide).

Energy dispersive X-ray analysis (EDAX)

The results of the X-ray microanalysis of proboscis hooks are given in Table 2 and Fig. 5. The common chemical elements for organic material (C, H, O, N) as well as the processing chemicals (Pd, Au, Ga) are present but not reported. The amount of sulfur in hooks was relatively high in hook tip and middle area in both adults and cystacanths but significantly lower in other parts of adult hooks. The amounts of phosphorus and calcium were generally lower in hook tip than in other hook parts, as well as consistently and markedly higher in adult than in cystacanth hooks.

Remarks

Batchvarov and Combes (1974) described *A. balkanicus* from one male and one female from the northern crested newt, *T. cristatus,* from a pond in southern Bulgaria. The host is a land-dwelling amphibian that breeds in ponds. They provided line drawings of the male specimen, the proboscis of male and female specimens showing no hook roots, measured the trunk, the proboscis, and the testes, counted 6 cement glands, and 10 rows of 5–6 proboscis hooks each. Their male and female specimens were reported to be 5.28 mm and 6.38 mm long, respectively, but their male's trunk (their fig. 1) measured only 3.3 mm in length according to the measurement scale. They also listed proboscis length to be 1.03 mm and 1.56 in the male and female specimens, respectively, but these proboscides (their fig. 2) measured only about 0.5 mm each according to the measurement scale. These inconsistencies might imply that they included the proboscis, neck, and bursa in their trunk measurements, as well as the neck in their proboscis measurements. Irrespective of their reported measurements, their line drawings suggest trunk and proboscides sizes of their specimens considerably smaller than in our specimens. The size of the anterior and posterior testes in their specimens was about the same, 0.63–0.64 × 0.19 mm but their fig. 1 shows a larger anterior testis than the posterior one. Batchvarov and Combes (1974) also described the root of the basal proboscis hook as "rudimentary" but we found roots to be prominent in all hooks. Unfortunately, it was not possible to investigate the original material studied by Batchvarov and Combes (1974).

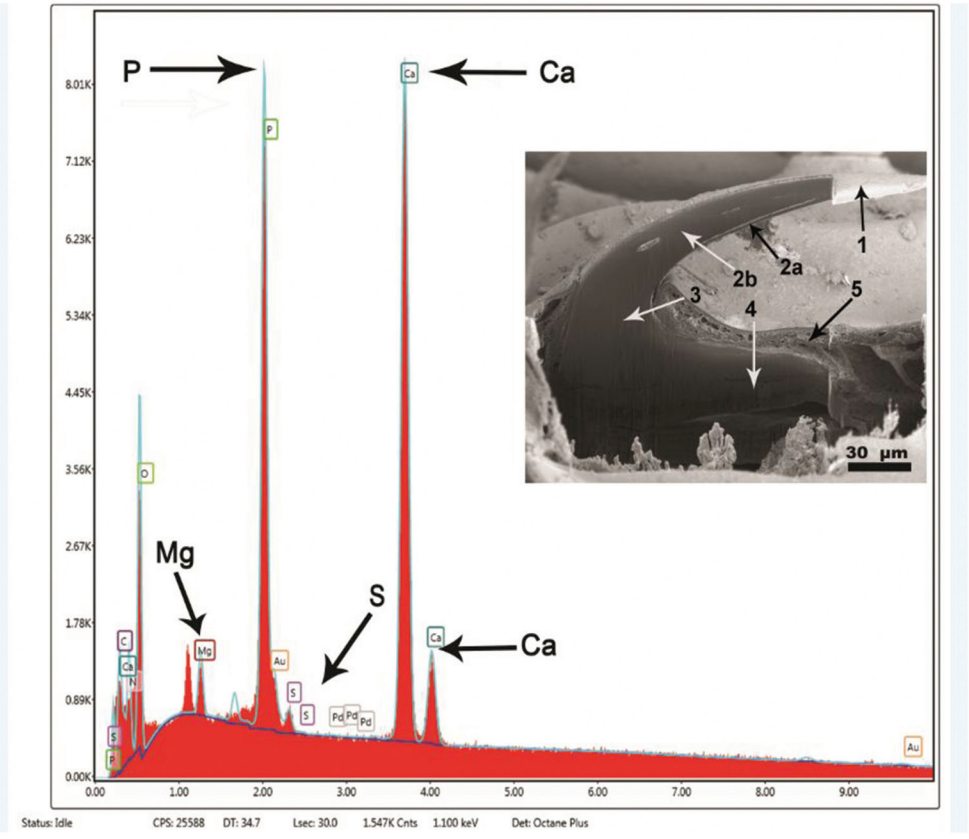
Molecular findings

Specimens collected from the olm *P. anguinus* and the cave ecomorph of *A. aquaticus* in Slovenia had completely identical sequences for all four genetic markers used in this study. Phylogenetic analyses and pairwise comparisons of sequences clearly demonstrated their affiliation to the genus *Acanthocephalus* and a high genetic similarity with *A. anguillae*, a common parasite of many species of European freshwater fishes (Fig. 6). All three nuclear markers (i.e., 18S rRNA gene, ITS, and 28S rRNA gene fragment) had identical sequences to the corresponding sequences of the herein used specimens of *A. anguillae* from Germany. Some genetic variability was observed only in the mitochondrial COI gene fragment for which the average uncorrected genetic distance equaled 2.1%. Considering that the COI gene is a highly variable marker on intra and interspecific level, the latter value is low and corresponds to genetic distances found within, rather than between, acanthocephalan species (e.g., Garcia-Varela and Pérez-Ponce de León 2008, Špakulová et al. 2011). On the other hand, a genetic distance between *Ac. an. balkanicus* and *Acanthocephalus nanus* Van Cleve, 1925 is about 10-fold higher (26.8%). Genetic distances based on the much more conserved nuclear 18S gene between our specimens and specimens of *A. anguillae* from previously published studies did not exceed 0.12% and are most likely due to sequencing errors.

Table 2. X-ray scans for a gallium cut hook of *Acanthocephalus anguillae balkanicus* subsp. n. for both the cystacanth and adult form. Cuts were made from the tip (1) of the hook to the area surrounding the base of the hook (5). Values correspond to the weight percent (wt %) of an element*. (see Fig. 5 for mapping cuts).

			Adult		
Element	Hook tip (1)	Mid area edge (2a)	Arch area of hook (3)	Internal base of hook (4)	Area surrounding hook base (5)
Phosphorus (P)	4.05	6.90	14.25	13.37	11.57
Sulphur (S)	11.22	4.43	0.59	0.80	1.45
Calcium (Ca)	7.26	12.34	33.86	30.69	27.29
			Cystacanth		
Phosphorus (P)	0.65	0.45	1.25	3.98	2.97
Sulphur (S)	7.27	4.78	8.03	5.00	7.12
Calcium (Ca)	0.15	0.01	0.40	0.55	0.63

*Common elements for living cells (C, H, O, N) as well as processing and coating elements (Pd, Au, Ga) are not included in the table.



Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C K	5.4	9.62	12.01	124.65	0.0159
N K	15.51	23.69	12.57	146.53	0.0199
O K	27.84	37.21	10.54	680.72	0.0444
Mg K	1.16	1.02	8.78	162.01	0.0071
P K	14.27	9.85	2.9	2076.67	0.1214
Au M	1.31	0.14	13.56	115.51	0.0157
S K	0.59	0.39	12.5	79.72	0.0049
Pd L	0.06	0.01	57.13	3.45	0.0004
Ca K	33.86	18.07	2.29	2784.88	0.3047

Fig. 5. The element scan (EDAX) at the middle arch area of a hook of an adult *Acanthocephalus anguillae balkanicus* specimen from *Proteus anguinus* Laurenti. The x-ray peak lines (K, L, or M) are listed for each element (first column; see periodic table of chemical elements). The last three columns (Error %, Net.Int, and K ratio) are the SEM TEAM-software correction factors.

DISCUSSION

Acanthocephalus anguillae balkanicus is definitely recognisable but the original description of Batchvarov and Combes (1974) from Bulgaria was far from comprehensive. In this paper, we provide measurements and description of all structures from a new and unique definitive host and from a new location, a cave in Slovenia. Our description also includes SEM micrographs and metal analysis of hooks. We report for the first time the size of hooks, neck, proboscis receptacle, lemnisci, and female reproductive system, as well as the shape of hook roots and female reproductive system from a larger number of specimens. We also report the finding of cystacanths from the first reported intermediate host, the cave ecomorph of the freshwater isopod crustacean *Asellus aquaticus*.

Our findings are especially interesting as the definitive host is an unusual amphibian, the olm, *Proteus anguinus*, the only exclusively cave-dwelling vertebrate in Europe, captured in the biologically highly diverse Postojna-Planina Cave System in Slovenia. Since olm's parasites from its natural environment are poorly known (Kostanjšek et al. 2017), our findings point to an additional health threat to this endangered species, listed also as a priority species under EU Habitats Directive, which might turn out to be important for its conservation. The only other known record of *Acanthocephalus anguillae balkanicus* is from another caudate amphibian, the northern crested newt, *Triturus cristatus* found in a distant location, a pond near village Pesnopoy in the Eastern Rhodope Mountains of southern Bulgaria (Batchvarov and Combes 1974). However, *T. cristatus* was recently found to be a complex of species (Wielstra and Arntzen 2011) and newts from southern Bulgaria have been described as a new species – the Balkan crested newt, *T. ivanbureschi* (see Wielstra et al. 2013). Thus, it is almost certain that acanthocephalan specimens described by Batchvarov and Combes (1974) were obtained from *T. ivanbureschi* rather than from *T. cristatus* sensu stricto. The two definitive host species of *Ac. an. balkanicus* therefore inhabit distant and unconnected water catchments, their distributional ranges do not overlap, and they live in distinct habitats. The olm is endemic to the subterranean waters of the Dinaric Karst (north western Balkan Peninsula), more specifically to the basin of the Soča River near Trieste in Italy, southern Slovenia, Croatia, and Bosnia and Herzegovina (Sket 1997). On the other hand, the Balkan crested newt is found in south-eastern Balkan Peninsula, i.e., most of Bulgaria, the eastern parts of Greece, Macedonia, and Serbia, as well as European Turkey, where it resides in various kinds of surface water bodies such as ponds, ditches, cisterns, and quarries (Wielstra et al. 2013). The great contrast in the habitats of both salamander species hosting the same acanthocephalan species is surprising but must be inclusive of involvement of the common intermediate host, *A. aquaticus*, which is distributed throughout most European freshwaters (Sket 1994). With such diversity in microhabitats, it is not unlikely that *Ac. an. balkanicus* may be found in other related settings elsewhere in Europe.

The element composition of the proboscis hooks of *Ac. an. balkanicus* is similar to those observed in other acanthocephalan species (see Heckmann et al. 2007, 2012, Brázova et al. 2014, Amin and Heckmann 2017). As expected from these studies, the proboscis hooks had a consistent level of the 3 key ions, i.e., calcium, phosphorus, and sulfur, with the latter predominantly concentrated in the cortical layer of hooks. It is assumed that the presence of sulfur ions is due to cysteine amino acids incorporated in a complex protein which is hardened by disulfide bonds (Heckmann et al. 2012).

Using X-ray diffraction Raynaud et al. (2002) demonstrated that the increased stability of a protein, such as in the proboscis hook, is due to the amount of disulfide bonds in the product. The outer hook layer is additionally hardened by calcium and phosphorous constituting the rigid calcium phosphate apatite, which is a similar compound to the one found in the enamel layer of mammalian teeth. In the herein investigated acanthocephalan, the calcium and phosphorus levels are clearly associated with the hardening of hooks in adults. The elemental profile of hooks but also spines has a taxonomic implication as it varies between acanthocephalan species and can thus be used as a fingerprint by which species can be diagnosed.

The presence of micropores in the body wall of *Ac. an. balkanicus* suggests that the whole trunk is involved in nutrient absorption, just like the trunk of practically all acanthocephalans that have been previously examined. Heckmann et al. (2013) have documented this phenomenon in 16 species of acanthocephalans and a few more have been identified since. The functional aspects of micropores in a few other acanthocephalan species including *Rhadinorhynchus ornatus* Van Cleave, 1918, *Polymorphus minutus* (Goeze, 1782), *Moniliformis moniliformis* (Bremser, 1811), *Macracanthorhynchus hirudinaceus* (Pallas, 1781), and *Sclerocollum rubrimaris* Schmidt et Paperna, 1978 were reviewed earlier by Amin et al. (2009). The micropore canals appear to be continuous with canalicular crypts that constitute a huge increase in external surface area implicated in nutrient uptake (Amin et al. 2009).

Resolving phylogenetic relationships within the genus *Acanthocephalus* is currently a challenge due to the surprisingly scarce molecular data available for its species and the ambiguity of several sequences available in GenBank. Some specimens are not identified to the species level and others are most likely misidentified. For example, our molecular analyses revealed that the specimen labelled as "*Acanthocephalus* sp." in Table 1 is most probably *Acanthocephalus lucii* (Müller, 1776) as its 18S gene sequence is identical to another specimen originally identified as *A. lucii*. It is also apparent from the phylogenetic tree in Fig. 6 that the specimen labelled as "*Acanthocephalus dirus*" (Van Cleve, 1931) was most likely misidentified and in reality belongs to the species *A. anguillae*. Assuming it was identified correctly evokes a non-monophyletic status of *A. anguillae* which we consider to be less probable. Most importantly, many of the publically available COI sequences for the genus in focus could be misleading due to the presence of COI pseudogene sequences which were

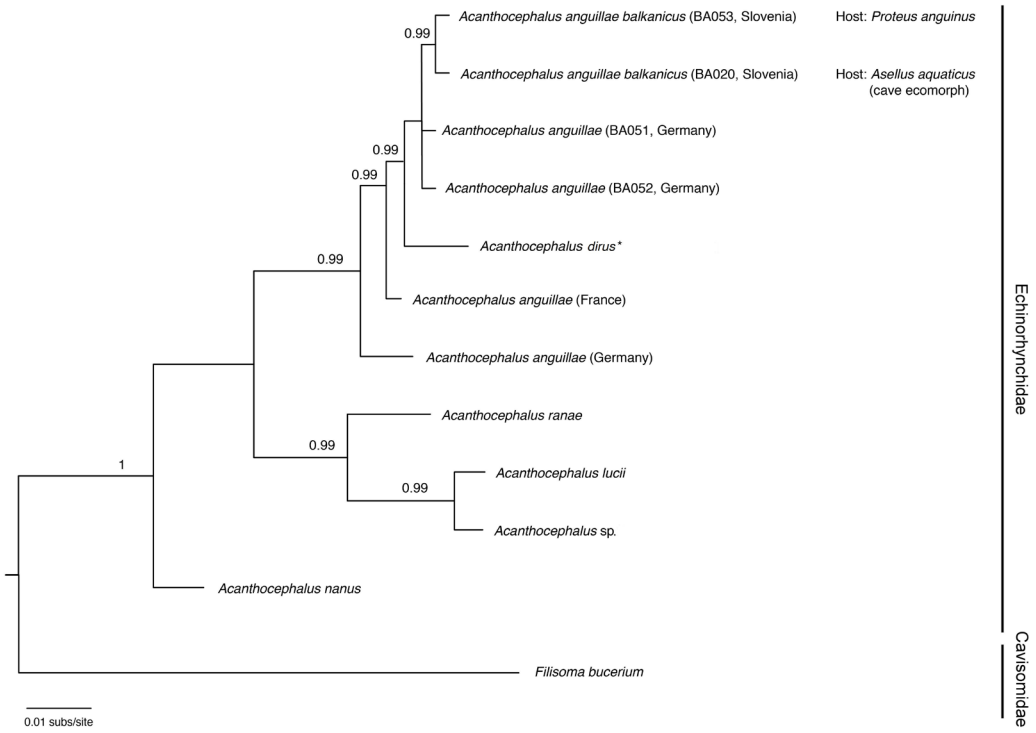


Fig. 6. Phylogenetic relationships within the genus *Acanthocephalus.* The tree was conducted using Bayesian inference on concatenated COI, 18S rRNA, ITS, and 28S rRNA gene sequences of taxa listed in Table 1. Only posterior probabilities exceeding 0.95 are shown. *Note that the *A. dirus* specimen was most likely misidentified and more probably belongs to *A. anguillae*.

shown and thoroughly discussed by Benesh et al. (2006). Without sequence chromatograms at hand, it is challenging to determine which sequence could be a pseudogene and therefore misleading.

Due to the above mentioned difficulties, a cautious assembly of our molecular dataset was crucial for a reliable inference of phylogenetic relationships within the genus *Acanthocephalus*. A close relationship of our specimens from cave-dwelling intermediate and definitive hosts to the common and widely distributed parasite *A. anguillae* was supported by high posterior probabilities. Their pairwise uncorrected genetic distances for the COI gene were minor and corresponded to those usually found on the intraspecific rather than the interspecific level (e.g., García-Varela and Pérez-Ponce de León 2008, Špakulová et al. 2011). This justifies the redescription of the morphologically and host-wise distinguishable *A. balkanicus* as a subspecies of *A. anguillae* rather than as a separate species. The phylogenetic tree in Fig. 6 further shows and supports by high posterior probabilities that *A. anguillae* is more related to *A. ranae* and *A. lucii* than to *A. nanus*. This is not surprising as the first two species are native to Europe just like *A. anguillae*, whereas the third species was found in Japan. Considering that there are 53 species of *Acanthocephalus* known worldwide (Amin 2013), our phylogenetic analysis is very basic. Molecular data of additional congeners are needed for further and more comprehensive phylogenetic and comparative analyses. We hope our molecular contribution will stimulate and help in future efforts of resolving phylogenetic relationships within this acanthocephalan genus after which the entire phylum of these intriguing parasitic animals was named.

As far as we are aware, the only other acanthocephalan species previously reported from a cave-bound host is *Neoechinorhynchus cylindratus* (Van Cleave, 1913) which was found infecting the intestine of the troglobitic northern cavefish *Amblyopsis spelaea* DeKay in a cave in Breckenridge County in Kentucky, USA (Nickol and Whittaker 1978). This acanthocephalan species is a common parasite of freshwater surface fishes in North America. The specimens from cavefish were morphologically indistinguishable from specimens found in surface waters. Nevertheless, Nickol and Whittaker (1978) discussed the possibility of parasite isolation in caves in light of the inability of troglobitic fauna to survive in surface waters. They proposed that such a barrier would promote parasite speciation underground. Nickol and Whittaker (1978) also proposed that the ostracod intermediate hosts of *N. cylindratus* would have been washed into caves during floods. This case may be comparable to that of the cave ecomorph of *A. aquaticus* and *Ac. an. balkanicus* in Slovenia. Still, the latter situation is highly intriguing as both definitive and intermediate hosts of this acanthocephalan are exclusively bound to cave life, which does not exclude the possibility that the entire life cycle of the parasite is being completed underground.

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Authors' declaration. O. Amin made the taxonomic decisions, wrote the manuscript, and created the line drawings. R. Heckmann created the SEM images and the EDAX analysis. Ž. Fišer discovered the parasites in the cave-dwelling hosts, wrote host-related parts of the manuscript, and contributed significant perspectives and opinions. V. Zakšek provided the molecular data and performed the molecular analysis. H. Herlyn collected and provided specimens of *A. anguillae* for molecular analysis. R. Kostanjšek helped with the collection of study material and provided SEM Fig. 4B. All authors critically reviewed the manuscript.

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