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Polystoma nacialtuneli n. sp. (Monogenea: Polystomatidae) from the eastern spadefoot, *Pelobates syriacus* (Pelobatidae) in Turkey

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Abstract

Polystoma nacialtuneli n. sp. is described from the urinary bladder of the eastern spadefoot, *Pelobates syriacus* from Turkey. This is the fifth polystome species known from Turkey and the third species in *Pelobates*. We show that this new parasite species can be distinguished from other polystome species in the area by a combination of characteristics, including parasite size and the shape and size of the hamuli. *Polystoma pelobatis* from *Pelobates cultripes* has a pair of well-developed hamuli, while *P. fuscus* from *Pe. fuscus* characteristically has a pair of underdeveloped hamuli barely larger than the marginal hooklets. *Polystoma nacialtuneli* n. sp. has well-developed hamuli that vary significantly in shape. Phylogenetic relationships of *P. nacialtuneli* n. sp. within *Polystoma*, supplemented with molecular divergences estimated from internal transcribed spacer 1 (ITS1) sequences, indicate that they are well separated from their closest relatives, i.e. *P. fuscus* and *P. pelobatis* from *Pe. fuscus* and *Pe. cultripes*, respectively.

Introduction

Polystomatids of anuran hosts in the Palaearctic realm are represented by the genera *Diplorchis* Ozaki, 1931 and *Polystoma* Zeder, 1800. In this geographical area *Polystoma* is represented by 18 species found in the anuran genera *Hyla*, *Pelobates*, *Pseudepidalea*, *Rana* and *Rhacophorus.* During a study of the helminth fauna of the eastern spadefoot *Pelobates syriacus* Boettger in Turkey some specimens were found to be infected with a new species of *Polystoma.* Two *Polystoma* species are known from the host *Pelobates* Wagler, namely *Polystoma fuscus* Biserkov & Hadjinikolova (1993) from *Pe. fuscus* (Laurenti) from Bulgaria and *P. pelobatis* Euzet & Combes, 1966 from *Pe. cultripes* (Cuvier) from France. Polystome species known from Turkey include *P. macrocnemis* Biserkov *et al.*, 2001 from *Rana macrocnemis* Boulenger, *P. skrjabini* Bukvarov, 1984 from *Hyla arborea* (Linnaeus), *P. viridis* Euzet, Combes & Batchvarov, 1974 from the frog *Pseudepidalea viridis* (Laurenti) (formerly known as *Bufo viridis* Boulenger) (see Yildirimhan *et al.*, 2006a, b). The specimens recovered from *Pe. syriacus* differed substantially from the other polystomes known to infect the host *Pelobates* and this is a formal description of the new species.

Materials and methods

Collection and examination of frogs

A total of 91 specimens of *Pe. syriacus* were collected from three localities in Turkey. Twenty-five specimens were collected from Bursa (40°16′N, 29°02′E) during May 1995, 24 specimens from the Edirne (41°51′N, 26°38′E) during May 2000, 19 specimens from the Seydişehir

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(37°20′N, 32°06′E) during July 2000, seven specimens during August 2002 and 16 specimens during June 2003.

Prior to dissection, frogs were anaesthetized by injecting them with 100 mg/kg body weight sodium pentobarbitone. Mature parasites were obtained from the urinary bladders and fixed under cover slip pressure for 24 h in 70% ethyl alcohol. Flattened specimens were stained in iron acetocarmine (Georgiev *et al.*, 1986), dehydrated, cleared and mounted in Entellan. Specimens were later remounted using Canada balsam.

Mounted parasites were examined using a Nikon (IMP, Boksburg, South Africa) E800 microscope fitted with a Nikon DXM1200 digital microscope camera connected to a PC. Measurements were taken using Eclipse network software (Nikon). All parasite measurements are in micrometres. Measurements are presented as the mean, followed by the range in parentheses and based on seven mature specimens.

Morphometrical comparison of the marginal hooklets

Marginal hooklet size and shape are of taxonomic importance when comparing polystomes. The protocol developed by Du Preez & Maritz (2006) was applied and a plot prepared.

Molecular analyses

Two polystome specimens from *Pe. syriacus* and one single individual from *Pe. cultripes* were analysed. We followed the molecular procedure described in Verneau *et al.* (2009) for DNA extractions and ITS1 amplifications. Polymerase chain reaction (PCR) was performed with Forward S1, 5′-ATTCCGATAACGAACGAGACT-3′ and Reverse IR8, 5′-GCTAGCTGCGTTCTTCATCGA-3′ primers (Sinnappah *et al.*, 2001; Kaci-Chaouch *et al.*, 2008) that anneal in the 18S rRNA and 5.8S rRNA genes, respectively. PCR products of approximately 850 bp were purified with the kit Wizard SV Gel and PCR Clean-Up System of Promega (Charbonnières, France) and sequenced with the Forward and Reverse primers by GATC Biotech (France). DNA sequences were subsequently edited and corrected using Sequencher^{TM} software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned using Clustal W (Thompson *et al.*, 1994) which is implemented in MEGA version 4 (Tamura *et al.*, 2007) with 15 other polystome sequences extracted from GenBank (table 1). After excluding indels and ambiguous characters, the maximum likelihood (ML) phylogenetic analysis was performed on 553 characters. A search for the best ML tree was done using the general time reversible $+\gamma$ model selected by the akaike information criterion in Modeltest (Posada & Crandall, 1998) and following a heuristic procedure under the tree bisection and reconnection branch swapping option with PAUP* 4.0b9 (Swofford, 2002). It was rooted with *Polystoma nearcticum*, according to Bentz *et al.* (2001) and Verneau *et al.* (2002). ML bootstrap support values were inferred with the same model of sequence evolution under the nearest neighbour interchange branch swapping option. Uncorrected pairwise divergences (p-distances) were also estimated within each pair of polystome taxa using PAUP* 4.0b9 to validate the taxonomic status of species.

Results

Levels of infection

Only host specimens from the sandy shores of Sigla Lake (Seydişehir) were infected. Of the 19 host specimens (13♂, 6♀) collected in July 2000, one male was infected with a single polystome (prevalence 5.3%); of the seven specimens (4♂, 3♀) collected in August 2002, one male was infected with two polystomes (prevalence 14.3%); and of the 16 specimens (9♂, 7♀) collected in June 2003, five males were infected with one polystome each, while one female was infected with three polystomes

Table 1. List of parasites included in the molecular analysis, their host species, country of origin and GenBank accession numbers.

Parasite species	Host species	Country of origin	Accession numbers for ITS1
Polystoma dawiekoki (1)	*Ptychadena anchietae*	Tanzania	AJ310405
Polystoma dawiekoki (2)	*Ptychadena anchietae*	South Africa	AJ310406
Polystoma fuscus	*Pelobates fuscus*	Bulgaria	AJ310401
Polystoma gallieni	*Hyla meridionalis*	France	AJ301687
Polystoma integerrimum (1)	*Rana temporaria*	France (Pyrénées Orientales)	AJ310411
Polystoma integerrimum (2)	*Rana temporaria*	France (Jura)	AJ301688
Polystoma integerrimum (3)	*Rana temporaria*	France (Jura)	AJ310410
Polystoma mangenoti	*Ptychadena superciliaris*	Ivory Coast	AJ310408
Polystoma marmorati	*Hyperolius m. marmoratus*	South Africa	AJ310496
Polystoma nacialtuneli (1)	*Pelobates syriacus*	Turkey	FR821517
Polystoma nacialtuneli (2)	*Pelobates syriacus*	Turkey	FR821518
Polystoma nearcticum	*Hyla versicolor*	USA	AJ301692
Polystoma occipitalis	*Hemisus marmoratus*	Ivory Coast	AJ301686
Polystoma pelobatis	*Pelobates cultripes*	France	FR821519
Polystoma sp.	*Cacosternum nanum*	South Africa	AJ310400
Polystoma umthakathi	*Natalobatrachus bonebergi*	South Africa	AJ301685
Metapolystoma brygoonis	*Ptychadena mascareniensis*	Madagascar	AJ310399

Table 2. Mean character differences inferred from comparisons of ITS1 sequences (553 aligned positions after excluding gaps and ambiguous characters).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Polystoma nacialtuneli* (1)																
2	*Polystoma nacialtuneli* (2)	0.000															
3	*Polystoma fuscus*	0.018	0.018														
4	*Polystoma pelobatis*	0.042	0.042	0.038													
5	*Polystoma occipitalis*	0.090	0.090	0.094	0.101												
6	*Polystoma umthakathi*	0.074	0.074	0.076	0.085	0.101											
7	*Polystoma marmorati*	0.080	0.080	0.081	0.090	0.103	0.009										
8	*Metapolystoma brygoonis*	0.090	0.090	0.090	0.096	0.085	0.101	0.099									
9	*Polystoma* sp.	0.081	0.081	0.081	0.089	0.105	0.020	0.025	0.103								
10	*Polystoma dawiekoki* (1)	0.087	0.087	0.087	0.094	0.031	0.099	0.098	0.085	0.098							
11	*Polystoma dawiekoki* (2)	0.087	0.087	0.087	0.094	0.031	0.099	0.098	0.085	0.098	0.005						
12	*Polystoma mangenoti*	0.098	0.098	0.101	0.112	0.045	0.116	0.114	0.098	0.116	0.043	0.040					
13	*Polystoma gallieni*	0.063	0.063	0.063	0.072	0.101	0.085	0.087	0.108	0.092	0.096	0.096	0.110				
14	*Polystoma nearcticum*	0.148	0.148	0.141	0.146	0.168	0.141	0.139	0.165	0.154	0.177	0.175	0.181	0.148			
15	*Polystoma integerrimum* (2)	0.089	0.089	0.087	0.089	0.114	0.112	0.110	0.114	0.116	0.118	0.118	0.128	0.085	0.136		
16	*Polystoma integerrimum* (3)	0.087	0.087	0.085	0.087	0.114	0.110	0.108	0.114	0.114	0.116	0.116	0.127	0.083	0.134	0.002	
17	*Polystoma integerrimum* (1)	0.089	0.089	0.087	0.089	0.116	0.112	0.110	0.116	0.116	0.118	0.118	0.128	0.085	0.137	0.007	0.005

(prevalence 38%, mean intensity 1.3). For the total sample the prevalence was 19.4 and mean intensity 1.4. In total 11 *Polystoma* specimens were collected with a maximum of three specimens found per host. Of these, one specimen was lost, one badly damaged and two used for molecular analyses. The description is thus based on seven sexually mature worms.

Description of Polystoma nacialtuneli *n. sp.*

Deposition of type specimens. Holotype (HKMUK 2011.2.28.1) and one paratype (HKMUK 2011.2.28.2) in the Parasitic Worms Collection, Natural History Museum, London; two paratypes (NMB-P326 and NMB-P327) deposited in the Parasitic Worm Collection, National Museum, Aliwal Street, 9300 Bloemfontein, South Africa; three paratypes deposited in the helminth collection of Uludag University, Museum of Zoology, Bursa, Turkey.

Type host. *Pelobates syriacus* Boettger, 1889.

Site. Urinary bladder.

Type locality. Seydişehir (37°20′N, 32°06′E).

Etymology. The species is named after Professor Naci Altunel, University of Uludag, Turkey who made significant contributions to the development of parasitology in Turkey.

Morphological characteristics. General characteristics (fig. 1) fit in with the generic characteristics for *Polystoma*. Body pyriform, tapering anteriorly. Body length 5067 (3412–7013), greatest width at the level of the testis 1815 (1228–2771), width at vagina 1230 (887–1631). Haptor 1472 (993–2061) long, 2190 (1476–3286) wide, with three pairs of laterally located cup-like suckers, 468 (303–658) diameter, hamuli and 16 marginal hooklets. Eyespots not observed in adults. False oral sucker 565 (470–768) with oral cavity subterminal. Pharynx pear shaped, 393 (319–521) long, 371 (275–507) wide. Intestine bifurcate, intestinal caeca join in haptor to form a haptoral anastomosis. Up to 33 (18–47) lateral diverticula and 15 (13–19) medial diverticula per side. Medial diverticula branched forming up to two anastomoses (fig. 2). Six of the specimens with two hamuli and the holotype with only one hamulus (fig. 1). Hamulus with shallow incision between the two roots (fig. 3A), length 410 (275–545), recurved point 45 (40–51) long (fig. 3A), small compared to other polystomes from *Pelobates*. Placement of marginal hooklets as for other polystomes; pairs 1 and 2 posteriormost between suckers 1; pairs 3, 4 and 5 at bases of suckers and pairs 6–8 anterior in haptor between suckers 3; no oncomiracidia were available and marginal hooklets were measured in mature specimens. Although the length for several hooklets was measurable, only three marginal hooklets 1 were in a flat orientation enabling us to prepare drawings. Hooklet 1, longest and largest with length 31 (30–32) (fig. 3B); hooklets 2–8 of equal length 24 (21–26) (fig. 3C). Testis single, large, follicular, post-ovarian, ventral and situated in mid body. Vas deferens widens slightly anteriorly forming a seminal vesicle, narrows to open at common genital bulb. Genital bulb armed with seven or eight genital spines (fig. 3D). Genital spine length 44 (42–45), curved with branched root on proximal end and sharp point distally, arranged in single file in the

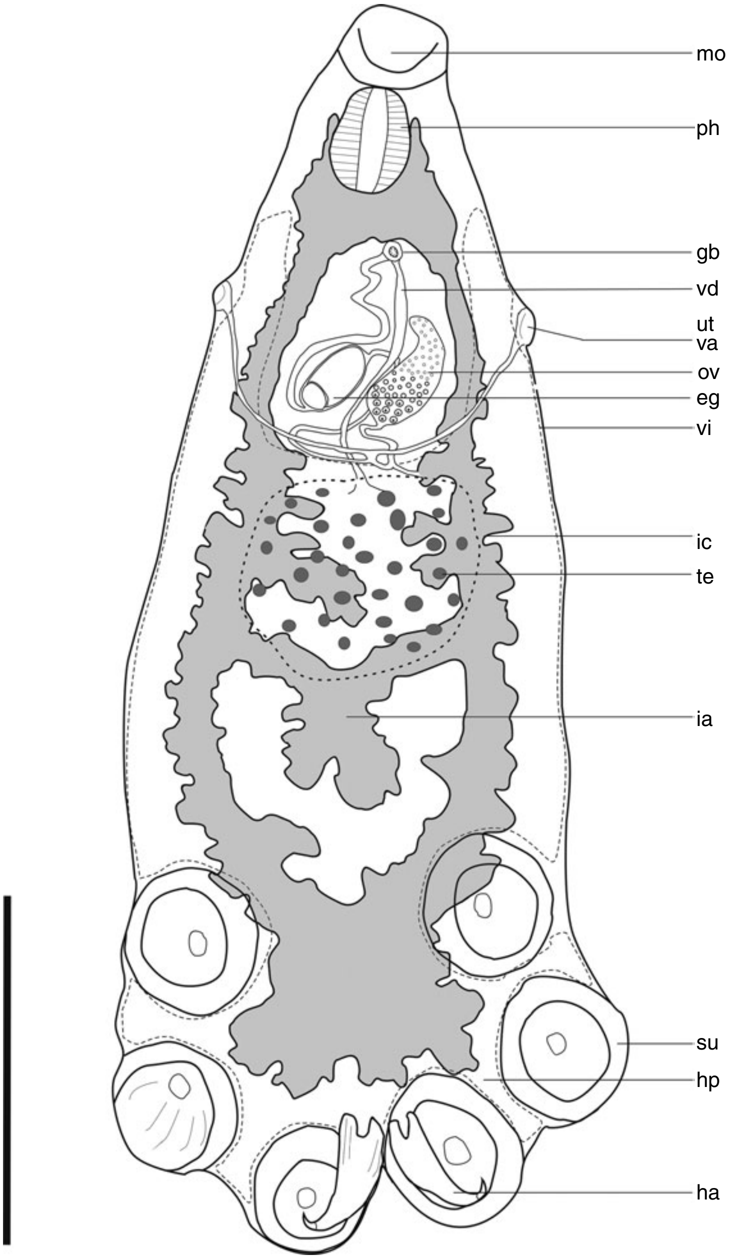

Fig. 1. *Polystoma nacialtuneli* n. sp., ventral view; dotted line indicates the outline of the vitelline system. Abbreviations: eg, egg; gb, genital bulb; ha, hamulus; hp, haptor; ia, intercaecal anastomosis; ic, intestinal caecum; mo, mouth; ov, ovary; ph, pharynx; su, sucker; te, testis; ut, uterus; va, vagina; vd, vas deferens; vi, vitellarium. Scale bar: 1 mm.

form of a bud, located anterior to ovary and posterior to intestinal bifurcation (fig. 1). Vaginae, two on lateral margins just anterior to the level of the ovary; vaginal ducts descend to respective vitelline ducts, main left and right vitelline ducts unite medially to form a vitelline reservoir, posterior duct connects to oviduct. Vitellarium follicular, diffuse, extending throughout most of the body except the area occupied by the ovary and uterus as well as around the haptoral suckers. Ovary pear-shaped, 522 (339–666) long, 268 (155–331) wide, one-third from anterior end of body (fig. 1). Oviduct leaves ovary in posterior direction, turns anteriorly and receives duct from vitelline reservoir, forms an ootype, surrounded by the Mehlis' gland. Uterus tubiform, relatively short and has a single yellowish-tan, operculated, oval-shaped egg 235 (234–236) long, 168 (156–180) wide *in utero* in four of the seven specimens examined. Oncomiracidium not known. Genito-intestinal canal arises just before entry of posterior vitelline duct, connecting oviduct to left caecum.

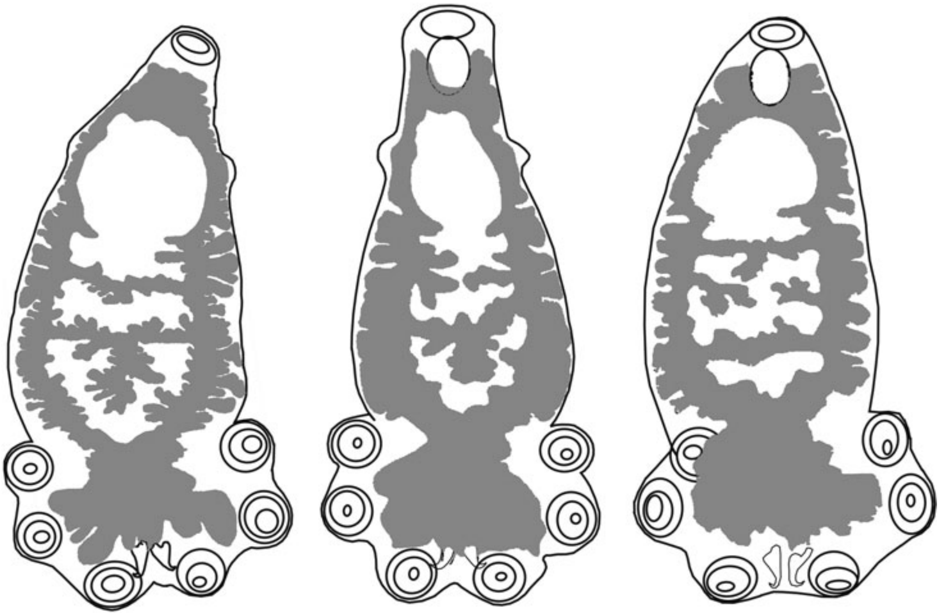

Fig. 2. *Polystoma nacialtuneli* n. sp., variation in intestinal arrangement.

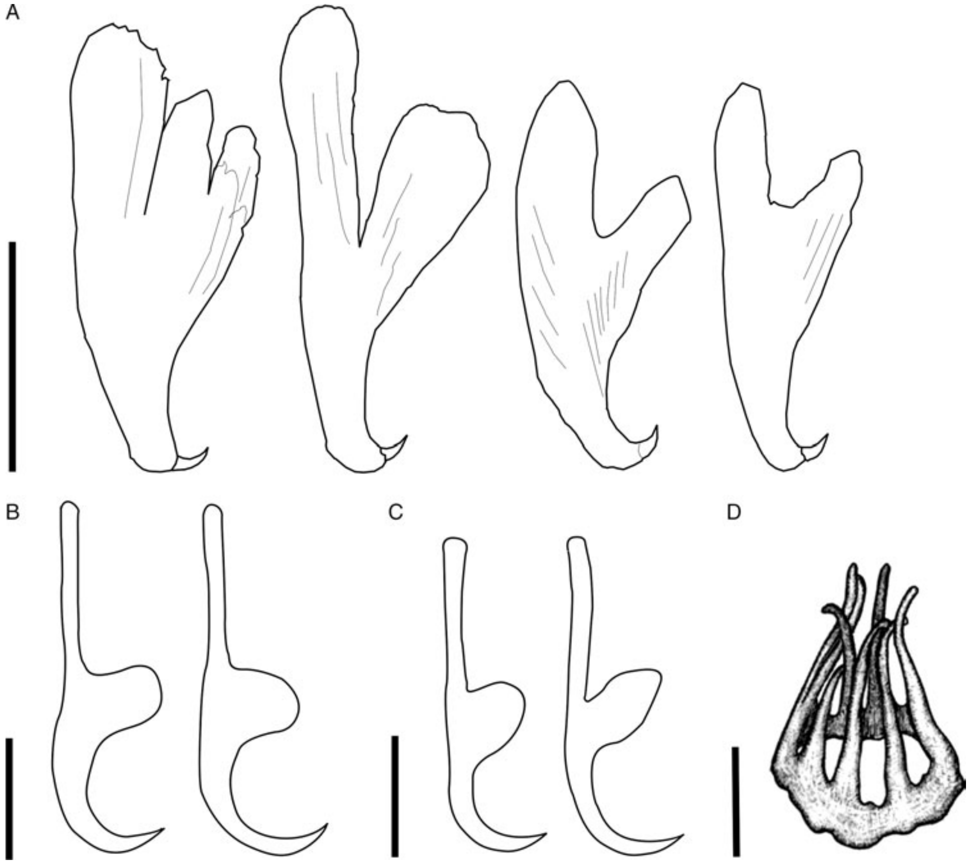

Fig. 3. *Polystoma nacialtuneli* n. sp. (A) hamuli; (B) marginal hooklets 1; (C) marginal hooklets 2–8; (D) genital spines. Scale bars: (A) 200 μm, (B) 10 μm, (C) 10 μm, (D) 20 μm.

Phylogenetic position

Regarding the genetic divergences estimated within each pair of polystomes, conspecific parasites of *Rana temporaria*, i.e. *P. integerrimum*, diverge from each other from 0.2 to 0.7% (table 2). Similarly, two conspecific individuals of *P. dawiekoki* which infests *Ptychadena anchietae* in allopatry show a divergence of 0.5%. On the other hand, the two most related African polystome species, namely *P. marmorati* of *Hyperolius m. marmoratus* and *P. umthakathi* of *Natalobatrachus bonebergi* (fig. 4) diverge from each other by 0.9%. Therefore, a threshold of about 1% divergence may be considered in order to assign individuals into separate species. Bentz *et al.* (2001) suggested that for *Polystoma* a 1% difference in ITS1 be considered as a separate species. Because the two individuals of *P. nacialtuneli* n. sp. diverge from their closest relative, i.e. *P. fuscus* of *Pe. fuscus*, by 1.8%, we should consider *P. nacialtuneli* n. sp. as a new polystome species. Figure 4 shows that *P. nacialtuneli* n. sp. falls within a clade of polystomes infesting archaeobatrachian hosts of family Pelobatidae, namely *Pe. cultripes*, *Pe. fuscus* and *Pe. syriacus*. This result suggests that these three polystome species may have coevolved with their host species, as is also the case for polystomes of *Ptychadena* in Africa (Bentz *et al.*, 2001).

Diagnosis

Polystoma nacialtuneli n. sp. differs from other polystomes known from *Pelobates* (table 3). Morphologically it is very similar to *P. fuscus* and body measurements overlap to some degree. The two species can, however, be separated on hamulus morphology. In *P. fuscus* the hamulus is totally underdeveloped and merely 15 μm long while in *P. nacialtuneli* n. sp. the hamulus is well developed with an average length of 410 μm. *Polystoma nacialtuneli* n. sp. differs from *P. pelobatis* in *Pe. cultripes* by a combination of characteristics. *Polystoma pelobatis* is double the size of *P. nacialtuneli* n. sp., with the smallest known specimen of *P. pelobatis* much larger than the largest *P. nacialtuneli* n. sp. Both species have prehaptoral intestinal anastomoses but *P. nacialtuneli* n. sp. has a maximum of two whereas *P. pelobatis* may have up to four. Hamulus length expressed as percentage of total body length is 8.2% (6.6–10.8%) for *P. nacialtuneli* n. sp. compared to 4.3% for *P. pelobatis*. In a plot of the products of the total length (a in fig. 5) and the width at the level of the guard (c in fig. 5) versus the product of the total length versus the length of a tangent between the tip of the blade to the guard (b in fig. 5) of marginal hooklet 1 as proposed by Du Preez & Maritz (2006), *P. nacialtuneli* n. sp. occupies a distinct position completely separated from *P. fuscus* and *P. pelobatis* (fig. 5).

Discussion

The newly described polystome species has a combination of characteristics that place it in the monogenean genus *Polystoma* Zeder, 1800, namely an attachment organ with three pairs of suckers, one pair of hamuli, intestinal caeca confluent to form a haptoral anastomosis, post-ovarian testis, short uterus, two vaginae and a follicular vitellarium. Because of limited interspecific variation in morphological characters used in identifying polystome

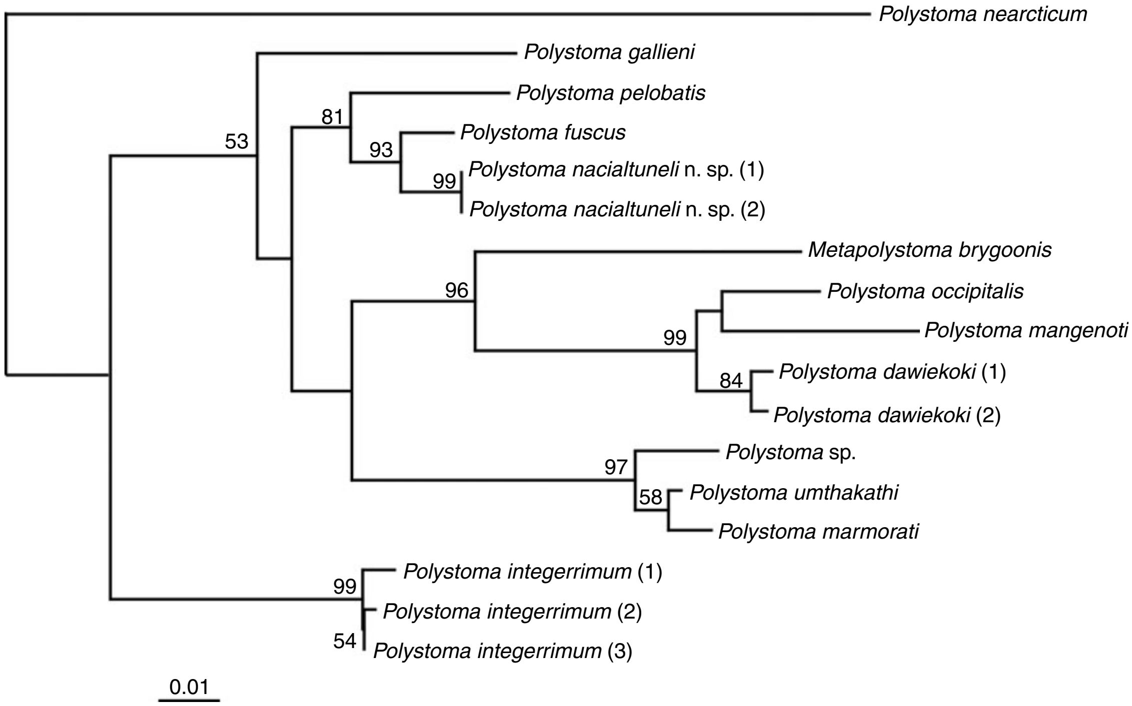

Fig. 4. Best maximum likelihood tree (score = 2182.70105) inferred from an analysis of 553 characters in the ITS1. Values along branches correspond to bootstrap proportions after 1000 replicates.

Table 3. Characteristics of *Polystoma* species known from *Pelobates* (all parasite measurements are in micrometres; measurements are presented as the mean, followed by the range in parentheses).

	P. nacialtuneli n. sp.	*P. fuscus*	*P. pelobatis*
Reference	Present study	Biserkov & Hadjinikolova (1993)	Euzet & Combes (1966) + own measurements
Host	*Pe. syriacus*	*Pe. fuscus*	*Pe. cultripes*
Country of origin	Turkey	Bulgaria	France
Number of specimens	7	39	5
Body length	5067 (3412–7013)	6574 (5405–8378)	10,347 (8200–11,809)
Maximum width	1815 (1228–2771)	2285 (1703–2756)	3488 (3019–4200)
Haptor length	1472 (993–2061)	1902 (1432–2432)	2484 (2113–2734)
Haptor width	2190 (1476–3286)	2667 (1432–3405)	3545 (3245–3698)
Sucker diameter	468 (303–658)	543 (380–693)	603 (520–691)
Hamulus length	410 (275–545)	15	446 (360–586)
Length marginal hooklet 1	31 (30–32)	24–32	39 (37–45)
False oral sucker width	565 (470–768)		619 (606–637)
Pharynx length	393 (319–521)	455 (440–812)	413 (397–436)
Pharynx width	371 (275–507)	451 (338–609)	404 (378–418)
Ovary length	522 (339–666)		1181 (1089–1231)
Ovary width	268 (155–331)		602 (539–653)
Number of genital spines	7	8 (6–9)	8
Length of genital spines	44 (42–45)	42–45	41 (39–42)
Egg length	235 (234–236)	237 (205–276)	246 (236–255)
Egg diameter	168 (156–180)	156 (147–169)	127 (123–132)
Anastomoses	0–2	Infrequent	0–4

species (Tinsley, 1973), much emphasis has been placed on host-specificity. This is also true for European polystomes. The first attempt to investigate the degree of host-specificity displayed by polystomes was conducted by Combes (1966). He conducted cross-infection experiments with two European polystome species. In cases where oncomiracidia became established in a substitute host tadpole, parasites progressively disappeared and no parasites were able to migrate to the urinary bladder. Combes (1968) conducted further cross-infection experiments and confirmed the strict host-specificity displayed by European polystomes. Euzet *et al.* (1974b) described *P. viridis* and showed that the new parasite was strictly host-specific and stated that, although the various

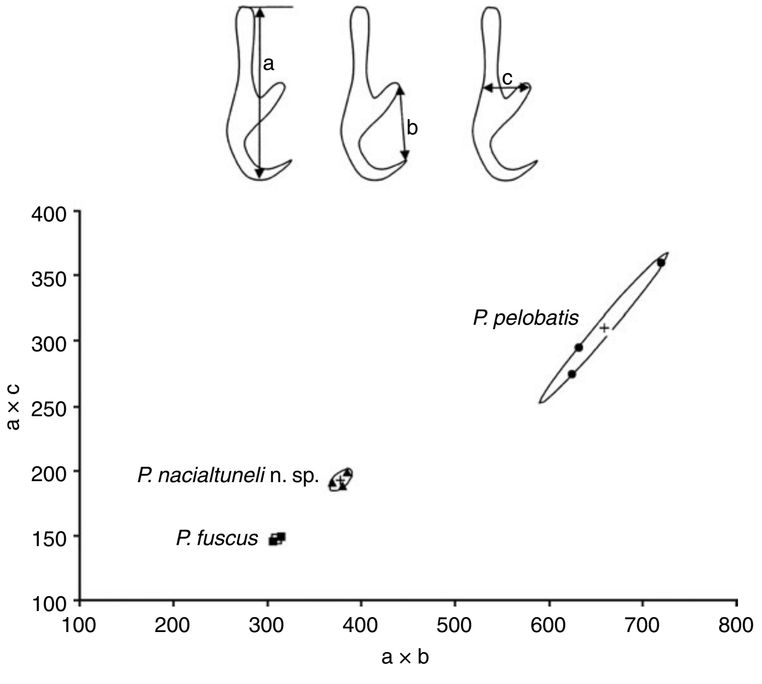

Fig. 5. Scatter diagram of a × b plotted against a × c for *Polystoma fuscus*, *P. pelobatis* and *P. nacialtuneli* n. sp. Measurements for *P. fuscus* were calculated from the drawings in the species description by Biserkov & Hadjinikolova (1993); and marginal hooklets for *P. pelobatis* and *P. nacialtuneli* n. sp. were measured from mounted mature specimens.

European polystomes must have a common ancestor, they have adapted to their 'new' hosts to such an extent that an exchange of hosts is impossible. Several other authors commented on the strict host-specificity of the Polystomatidae and, in particular, the genus *Polystoma* (Tinsley, 1973, 1974; Euzet *et al.*, 1974a; Bourgat & Salami-Cadoux, 1976; Combes & Channing, 1979; Murith, 1981, 1982; Kok & Van Wyk, 1986; Kok & Du Preez, 1987; Du Preez & Kok, 1992, 1993, 1997). This high degree of host-specificity has led to the acceptance that host species identity plays a key role in identifying a species.

Although the hamuli of *P. nacialtuneli* n. sp. are on average marginally smaller that those for *P. pelobatis* (410 for *P. nacialtuneli* n. sp. and 446 for *P. pelobatis*), the relative size of the hamulus expressed as a percentage of the total body length is double that of *P. pelobatis*. Since the hamuli for *P. fuscus* are very small (15), Biserkov & Hadjininikolova (1993) compared this species with all anuran polystomes lacking hamuli, namely *Eupolystoma*, *Riojatrema* and *Pseudodiplorchis,* and concluded that, apart from the hamulus, *P. fuscus* fits within the genus *Polystoma*.

The Polystomatidae is an ancient group of parasites tracking the evolutionary history of the first aquatic tetrapods following the Actinopterygii–Sarcopterygii transition in the Palaeozoic age (Verneau *et al.*, 2002). Bentz *et al.* (2001) hypothesized that the genus *Polystoma* had its origin in South America, from where it spread to Europe and to Africa. A strongly supported hypothesis is that the ancestor of *Pelobates* carried *Polystoma* into Africa. They proposed that this invasion could have taken place during the Miocene when, for a short period, Spain and Morocco were connected. This period matches the molecular calibrations by Bentz *et al.* (2001). Of the four currently recognized *Pelobates* species, only *Pe. varaldii* Pasteur & Bons is known from the African continent, where it occurs in Morocco. The other three, namely *Pe. cultripes, Pe. fuscus* and *Pe. syriacus* are all known from Europe. All three European species harbour closely related *Polystoma* species. Further recovery of a polystome in *Pe. varaldii* will be very significant as it would provide an opportunity to test the hypothesis that *Polystoma* was introduced to Africa through ancestral *Pelobates*.

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