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Molecular insight into systematics, host associations, life cycles and geographic distribution of the nematode family Rhabdiasidae ☆

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ABSTRACT

Rhabdiasidae Railliet, 1915 is a globally distributed group of up to 100 known species of nematodes parasitic in amphibians and reptiles. This work presents the results of a molecular phylogenetic analysis of 36 species of Rhabdiasidae from reptiles and amphibians from six continents. New DNA sequences encompassing partial 18S rDNA, ITS1, 5.8S rDNA, ITS2 and partial 28S rDNA regions of nuclear ribosomal DNA were obtained from 27 species and pre-existing sequences for nine species were incorporated. The broad taxonomic, host and geographical coverage of the specimens allowed us to address long-standing questions in rhabdiasid systematics, evolution, geographic distribution, and patterns of host association. Our analysis demonstrated that rhabdiasids parasitic in snakes are an independent genus sister to the rest of the Rhabdiasidae, a status supported by life cycle data. Based on the combined evidence of molecular phylogeny, morphology and life cycle characteristics, a new genus *Serpentirhabdias* gen. nov. with the type species *Serpentirhabdias elaphe* (Sharpilo, 1976) comb. nov. is established. The phylogeny supports the monophyly of *Entomelas* Travassos, 1930, *Pneumonema* Johnston, 1916 and the largest genus of the family, *Rhabdias* Stiles and Hassall, 1905. DNA sequence comparisons demonstrate the presence of more than one species in the previously monotypic *Pneumonema* from Australian scincid lizards. The distribution of some morphological characters in the genus *Rhabdias* shows little consistency within the phylogenetic tree topology, in particular the apical structures widely used in rhabdiasid systematics. Our data suggest that some of the characters, while valuable for species differentiation, are not appropriate for differentiation among higher taxa and are of limited phylogenetic utility. *Rhabdias* is the only genus with a cosmopolitan distribution, but some of the lineages within *Rhabdias* are distributed on a single continent or a group of adjacent zoogeographical regions. *Serpentirhabdias*, *Entomelas* and *Pneumonema* show rather strict specificity to their host groups. The evolution of the Rhabdiasidae clearly included multiple host switching events among different orders and families of amphibians as well as switching between amphibians and squamatan reptiles. Only a few smaller lineages of *Rhabdias* demonstrate relatively strict associations with a certain group of hosts.

1. Introduction

The cosmopolitan family Rhabdiasidae Railliet, 1915 includes up to 100 known nematode species parasitic in amphibians and reptiles. All share some morphological characters (Lhermitte-Vallarino et al., 2005) but the most remarkable feature of rhabdiasids is the regular alternation of parasitic and free-living generations (heterogony) in life cycles, with the exception of only one species, *Chabirenia cayennensis* Lhermitte-Vallarino, Bain, Deharo, Bertani, Voza, Attout & Gaucher, 2005.

Details of the cephalic morphology (presence/absence of lips and pseudolabia and their arrangement), the size of the buccal capsule and the presence/absence of certain cuticular structures (e.g., cuticular spines, pores or crests) have been traditionally among the morphological characters most commonly used for differentiation among rhabdiasid genera (Travassos, 1930; Sharpilo, 1976; Baker, 1980; Lhermitte-Vallarino et al., 2005; Kuzmin, 2013).

The systematics of the Rhabdiasidae, particularly at the generic level, has been unstable due in large part to the uncertain diagnostic value of various morphological characters used by different authors. Many authors have emphasised that due to the high level

☆ New nucleotide sequence data reported in this paper are available in GenBank under accession numbers KF999588–KF999614.

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of morphological uniformity among rhabdiasid species, and particularly those belonging to *Rhabdias* Stiles and Hassall, 1905, the differentiation of species in the family is often difficult (Chu, 1936a; Baker, 1978; Kuzmin et al., 2007). An insufficient number of reliable morphological characters has prohibited the construction of morphology-based phylogenetic hypotheses and the lack of a robust phylogeny has prevented the assessment of the utility of morphological characters used in the taxonomy of rhabdiasids.

Despite the unique biology of Rhabdiasidae, peculiarities of their life cycles (Anderson, 2000) have not been used as characters in their systematics or phylogeny. Heterogony is considered to be present in life cycles of all rhabdiasids. It is the only mode of development found in *Rhabdias* spp. parasitising amphibians (e.g., Leuckart, 1865; Metchnikoff, 1865; Baker, 1979; Kuzmin, 1997; Langford and Janovy, 2009; Junker et al., 2010), as well as in *Rhabdias* parasitic in lizards (Chabaud et al., 1961; Lhermitte-Vallarino and Bain, 2004; Lhermitte-Vallarino et al., 2009, 2010). Only heterogonic life cycles were also observed in members of the genera *Entomelas* Travassos, 1930 (see Seurat, 1920; Kuzmin, 2013) and *Pneumonema* Johnston, 1916 (see Ballantyne, 1991), parasites of lizards. However, *Rhabdias* from snakes are characterised by the presence of both heterogony and homogony in their life cycles. This combination of direct and indirect development has now been reported for several *Rhabdias* parasitising snakes, namely *Rhabdias fuscovenosa* Railliet, 1899, *Rhabdias elaphe* Sharpilo, 1976, *Rhabdias agkistrodonis* Sharpilo, 1976 and *Rhabdias eustreptos* McCallum, 1921 (Chu, 1936b; Kuzmin, 1999, 2013; Kuzmin and Miskov, 1999; Langford and Janovy, 2009).

While the majority of rhabdiasid genera demonstrate rather strict specificity to their hosts (e.g., snakes or lizards), representatives of the largest genus *Rhabdias* (up to 90 known species) are found in a wide range of hosts that include apodan, anuran and caudatan amphibians as well as squamatan reptiles (Sauria and Serpentes). Baker (1984) suggested that rhabdiasids originated in amphibians with a subsequent acquisition of these parasites by reptiles. The phylogenetic relationships of rhabidiasids from reptiles are unknown and these worms do not form a morphologically distinct lineage. Moreover, parasites of reptiles are scattered in all of the rhabdiasid genera, raising the possibility that host switching might have occurred several times in the evolutionary history of the family.

Molecular phylogenetics has become a common, highly valuable tool that is particularly useful in cases when morphology is not sufficient for phylogenetic reconstruction. However, none of the molecular analyses of the Rhabdiasidae published thus far have included a phylogenetic examination of a broad set of taxa from various hosts and continents. Several studies that have utilised DNA sequences in order to distinguish among closely related species of *Rhabdias* (Tkach et al., 2006; Kuzmin et al., 2007; Dare et al., 2008; Dubey and Shine, 2008; Junker et al., 2010; Cipriani et al., 2012) and the four publications that included more extensive phylogenetic reconstruction (Dare et al., 2008; Dubey and Shine, 2008; Cipriani et al., 2012; Langford and Janovy, 2013) suffered from low taxon sampling and limited geographic representation. For example, Dare et al. (2008) included a tree that contained only four species of *Rhabdias* from frogs and toads from three different continents, limiting our understanding of the overall relationships among members of this genus. Subsequent works have provided somewhat more robust, if incomplete, analyses. Dubey and Shine (2008) developed a phylogenetic tree largely comprised of *Rhabdias* collected from Australian amphibians as well as some Neotropical and North American species. Cipriani et al. (2012) showed interrelationships among *Rhabdias esculentarum* Cipriani, Mattiucci, Paoletti, Santoro & Nascetti, 2012, *Rhabdias bufonis* (Schrank, 1788) and *Rhabdias sphaerocephala* Goodey, 1924, all parasitic in European anuran amphibians. Langford and Janovy (2013) provided a molecular phylogeny for six species of *Rhabdias* parasitic in North American amphibians and snakes that allowed evolutionary prospective on parasite biology and host specificity.

Rhabdiasid taxonomy has been resurgent in recent years, with over 40 new species described since 2000. However, understanding of life cycle evolution, phylogenetic interrelationships and the nature of host associations remains limited.

The systematics of the family also remains confused with substantial uncertainty about the status and composition of genera and the systematic position of certain species.

We generated novel sequence data for the nuclear rRNA gene family for 27 rhabdiasid taxa and combined them with published data (Kuzmin et al., 2007; Dubey and Shine, 2008) to produce the most comprehensive phylogenetic analysis of Rhabdiasidae thus far.

2. Materials and methods

2.1. Sampling and morphological studies

Specimens of 27 rhabdiasid taxa were obtained from various groups of hosts on different continents (Table 1). Most of the nematodes were collected by the authors from freshly euthanised or frozen hosts. When required, appropriate Institutional Animal Care and Use Committee (IACUC) protocols were obtained. Specimens of *Rhabdias bermani* Rausch, Rausch & Atrashkevich, 1984 and *Rhabdias* cf. *bufonis* from the Magadan region, *Rhabdias bulbicauda* Sarkar & Manna, 2004 from Nepal, *Rhabdias* cf. *africanus* from Nigeria, *Rhabdias nicaraguensis* Bursey, Goldberg & Vitt, 2007 from Costa Rica, *Rhabdias elegans* Gutierrez, 1945 from Argentina and *Rhabdias* cf. *joaquinensis* 3 from South Carolina were kindly provided by our colleagues Olga Lisitsyna (Institute of Zoology, Ukrainian National Academy of Sciences, Kiev, Ukraine), Gennady Atrashkevich (Institute of Biological Problems of the North, Russian Academy of Sciences, Magadan, Russia), Sheekanta Poudel (University of North Dakota, USA), Martins Aisien (University of Benin, Nigeria), Eric Pulis (University of Southern Mississippi, USA), Agustín Jiménez (Southern Illinois University, USA), and Stephen Greiman (University of North Dakota).

Names of nematodes, their hosts and geographic localities are provided in the Table 1. For morphological studies and scanning electron microscopy the worms were killed with hot saline or hot 70% ethanol and fixed in 70% ethanol.

For light microscopical examination and taxonomic identification, specimens were cleared in glycerol by gradual evaporation from a 5% solution of glycerol in 70% ethanol (EtOH), or in phenol-glycerol (3:1) solution.

Nematodes used for scanning electron microscopy (SEM) were dehydrated in a graded series of ethanol and acetone, and dried using hexamethyldisilazane (HMDS) (Ted Pella, Inc., Redding, CA, USA) as transition fluid. Dry specimens were mounted on stubs, coated with gold–palladium and examined using a Hitachi 4700 scanning electron microscope (Hitachi USA, Mountain View, CA, USA) at an accelerating voltage of 10–15 kV.

2.2. DNA extraction, gene amplification and sequencing

For molecular analysis, live worms recovered from the host were rinsed thoroughly in saline, and fixed in 70% or 95% EtOH. New DNA sequences have been obtained for 31 rhabdiasid taxa. Due to the small size of the nematodes, in most cases the entire specimen was used for DNA extraction upon morphological identification. Genomic DNA was extracted from single specimens of worms according to Tkach and Pawlowski (1999) or using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). DNA

Table 1
Species of Rhabdiasidae used in this study, host species, geographical origin of material and GenBank accession numbers for corresponding sequences.

Nematode taxa	Host species	Geographic origin	GenBank Accession No.
Rhabditoides regina	Free-living	Not reported	EF990726
Entomelas entomelas	*Anguis fragilis*	Near Kiev, Ukraine	KF999592[a]
Entomelas kazakhstanica	*Pseudopus apodus*	Crimea, Ukraine	KF999597[a]
Entomelas ophisauri	*Pseudopus apodus*	Crimea, Ukraine	KF999595[a]
Entomelas dujardini	*Anguis fragilis*	Near Kiev, Ukraine	KF999591[a]
Entomelas sp.	*Ophisaurus* sp.	Near Gainesville, Florida, USA	KF999601[a]
Pneumonema tiliquae	*Tiliqua scincoides*	Townsville, Queensland, Australia	KF999611[a]
Pneumonema sp. 1	*Tiliqua scincoides*	Charleville, Queensland, Australia	KF999603[a]
Pneumonema sp. 2	*Cyclodomorphus gerrardii*	Mt Glorious National Park, Queensland, Australia	KF999612[a]
Rhabdias ambystomae	*Ambystoma maculatum*	Pigeon Lake, Wisconsin, USA	KF999590[a]
Rhabdias americanus	*Anaxyrus americanus*	Pigeon Lake, Wisconsin, USA	KF999589[a]
Rhabdias bakeri	*Lithobates sylvatica*	Nelson CO., North Dakota, USA	DQ264770
Rhabdias bermani	*Salamandrella keyserlingii*	Magadan Region, Russia	KF999610[a]
Rhabdias bufonis	*Rana temporaria*	Kiev, Ukraine	KF999593[a]
Rhabdias cf. *bufonis* 1	*Bombina bombina*	Poltava region, Ukraine	KF999606[a]
Rhabdias cf. *bufonis* 2	*Rana amurensis*	Magadan Region, Russia	KF999609[a]
Rhabdias bulbicauda	*Bufo* sp.	Pokhara, Nepal	KF999600[a]
Rhabdias cf. *africanus*	*Hylarana galamensis*	Nigeria	KF999598[a]
Rhabdias elegans	*Bufo* sp.	Argentina	KF999604[a]
Rhabdias joaquinensis	*Lithobates blairi*	Reelfoot Lake, Tennessee, USA	KF999594[a]
Rhabdias cf. *joaquinensis* 1	*Lithobates clamitans*	Rome, Georgia, USA	KF999608[a]
Rhabdias cf. *joaquinensis* 2	*Lithobates blairi*	Commercial supplier, USA	KF999602[a]
Rhabdias cf. *joaquinensis* 3	*Hyla* sp.	Isle of Palms, near Charleston, South Carolina, USA	KF999607[a]
Rhabdias kongmonthaensis	*Polypedates leucomystax*	Kong Mong Tha village, Kanchanaburi Province, Thailand	KF999599[a]
Rhabdias pseudosphaerocephala	*Rhinella marinus*	City of Leon, Leon Province, Nicaragua	DQ845737
Rhabdias ranae	*Lithobates pipiens*	Nelson Co., North Dakota, USA	DQ264766
Rhabdias nicaraguensis	*Norops* sp.	Área de Conservación Guanacaste, Costa Rica	KF999605[a]
Rhabdias rubrovenosa	*Bufotes viridis*	Rivne, Ukraine	KF999596[a]
Rhabdias sphaerocephala	*Bufo bufo*	Kiev, Ukraine	DQ845739
Rhabdias sp. II A1	*Rhinella schneideri*	Campinas city, Brazil (Dubey and Shine, 2008)	EU836870
Rhabdias cf. *hylae* VII A1	*Litoria alboguttata*	Queensland, Australia (Dubey and Shine, 2008)	EU836874
Rhabdias cf. *hylae* VI A2	*Platyplectrum ornatum*, *Litoria fallax*	Queensland, Australia (Dubey and Shine, 2008)	EU836868
Rhabdias cf. *hylae* IV–V A1	*Litoria caerulea*, *L. nasuta*, *L. rothii*, *L. alboguttata*, *L. novaehollandiae*	Northern Territory, Queensland, Australia, (Dubey and Shine, 2008)	EU836866
Rhabdias cf. *hylae* V A2	*Litoria pallida*	Queensland, Australia (Dubey and Shine, 2008)	EU836863
Serpentirhabdias fuscovenosa	*Natrix natrix*	Lesniki, Kiev region, Ukraine	KF999588[a]
Serpentirhabdias cf. *fuscovenosa*	*Nerodia erythrogaster*	Reelfoot Lake, Tennessee, USA	KF999613[a]
Serpentirhabdias elaphe	*Zamenis longissimus*	Zakarpatska oblast, Ukraine	KF999614[a]

[a] New sequences generated by this study.

fragments spanning the 3′ end of 18S ribosomal DNA (rDNA) gene, internal transcribed spacer (ITS) 1, 5.8S rDNA, ITS2, 5′ end of the 28S rDNA gene (including variable domains D1–D3) were amplified by PCR on an Eppendorf Master Gradient or Eppendorf EP Gradient thermal cyclers.

PCRs were performed in a total volume of 50 μl containing 41 μl of H_2O, 5 μl of Taq buffer, 1 μl of dNTP at a concentration of 10 pM/μl, 1 μl of each primer at a concentration 10 pM/μl, 0.25 μl of Eppendorf or 5 Prime Taq polymerase at a concentration 5 units/μl and 1–1.5 μl of template genomic DNA (gDNA) extract. The thermocycling profile was as follows: 2 min denaturation hold at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 53 °C, 2 min at 72 °C, and a 7 min extension hold at 72 °C.

The Rhabdiasidae-specific forward primer ritf (5′-GCGGCTTA ATTTGACTCAACACGG-3′) and the universal reverse primer 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) were used for both amplification and sequencing. Additionally, internal forward primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), 300R (5′-CAACTTTCCCTCACGG-TACTTG-3′) and ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′) were used for sequencing.

PCR products were purified using Qiagen Qiaquick™ columns or Exo-Sap IT PCR Product Clean-up kit from Affymetrix (USA) and sequenced directly on an ABI Prism 3100™ automated capillary sequencer using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) according to the manufacturer's protocols. Contiguous sequences were assembled and edited using Sequencher™ ver. 4.1.1 (GeneCodes Corp., USA) and submitted to GenBank. Accession numbers are provided in the Table 1.

2.3. Sequence alignment and phylogenetic analyses

The new sequences and sequences obtained from GenBank (Table 1) were aligned initially with the aid of ClustalW as implemented in the BioEdit program, version 7.0.1 (Hall, 1999). The alignments were then manually refined using BioEdit, saved in FASTA format and imported into the MacClade ver. 4.02 software (http://macclade.org/macclade.html). Upon selection of the exclusion sets the alignments were saved in NEXUS format for the subsequent analyses. Positions with ambiguous alignment were excluded from the analysis. Three distinct alignments, I–III, were prepared and three analyses were run.

Alignment I was based only on partial 28S rDNA gene sequences and included the free-living rhabditoid nematode *Rhabditoides regina* (Schulte & Poinar, 1991) as the outgroup and ingroup taxa that represented major lineages within the Rhabdiasidae. The alignment was trimmed to the length of the sequence of *R. regina* available in the GenBank.

Upon removal of the rhabditid outgroup, Alignment II used the longest sequences encompassing the 3′ end of the 18S rDNA gene – ITS1 – the 5.8S rDNA gene – ITS2 and the 5′ end of the 28S rDNA gene. It included *Serpentirhabdias fuscovenosa* comb. nov. from snakes as an outgroup, five species of *Entomelas*, three species of *Pneumonema* and only seven species of *Rhabdias* from amphibians. The purpose of this dataset and analysis was to test the monophyly of the remaining rhabdiasid genera and reveal internal interrelationships in the smaller genera *Entomelas* and *Pneumonema*.

Alignment III also used sequences spanning the 3′ end of the 18S rDNA gene – ITS1-5.8S rDNA gene – ITS2 and the 5′ end of the 28S rDNA gene. However, it was somewhat shorter than the Alignment II because it had to be trimmed to the length of the sequences published by Dubey and Shine (2008). This alignment and the corresponding analysis included the sequence of *Pneumonema tiliquae* Johnston, 1916 as an outgroup and sequences of all available species of *Rhabdias* other than those parasitic in snakes. The purpose of this alignment and analysis was to infer in detail the interrelationships within this largest rhabdiasid genus while preserving as many alignment positions as possible in the analysis.

Phylogenetic analysis was carried out using Bayesian inference (BI) as implemented in the MrBayes program (ver. 3.1). The Bayesian analyses were run with the following nucleotide substitution model settings: lset nst = 6, rates = invgamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, that correspond to a general time reversible (GTR) model including estimates of the proportion of invariant sites (I) and gamma (G) distributed among-site rate variation. The nucleotide substitution model was determined using MrModelTest 2.3 software. Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations for the first analysis and for 3,000,000 generations for the second and third analyses, log-likelihood scores plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burnin" parameter at 250,000 and 500,000 generations, accordingly. Trees were visualised using the FigTree ver. 1.4 software (Rambaut, A. 2012. Molecular evolution, phylogenetics and epidemiology: FigTree. URL: http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Molecular phylogeny

Alignment I comprised a total of 810 sites, of which 807 could be aligned unambiguously. The Bayesian analysis of Alignment I produced a phylogenetic tree shown in Fig. 1. Although the sequence of *Rhabditoides* was the closest to the Rhabdiasidae among all sequences presently available in GenBank, it still was very divergent from the sequences in the ingroup. Due to the high level of genetic divergence between the outgroup (*R. regina*) and ingroup taxa, the branch support in the first analysis was overall low (Fig. 1). Nevertheless, it revealed a major evolutionary split within

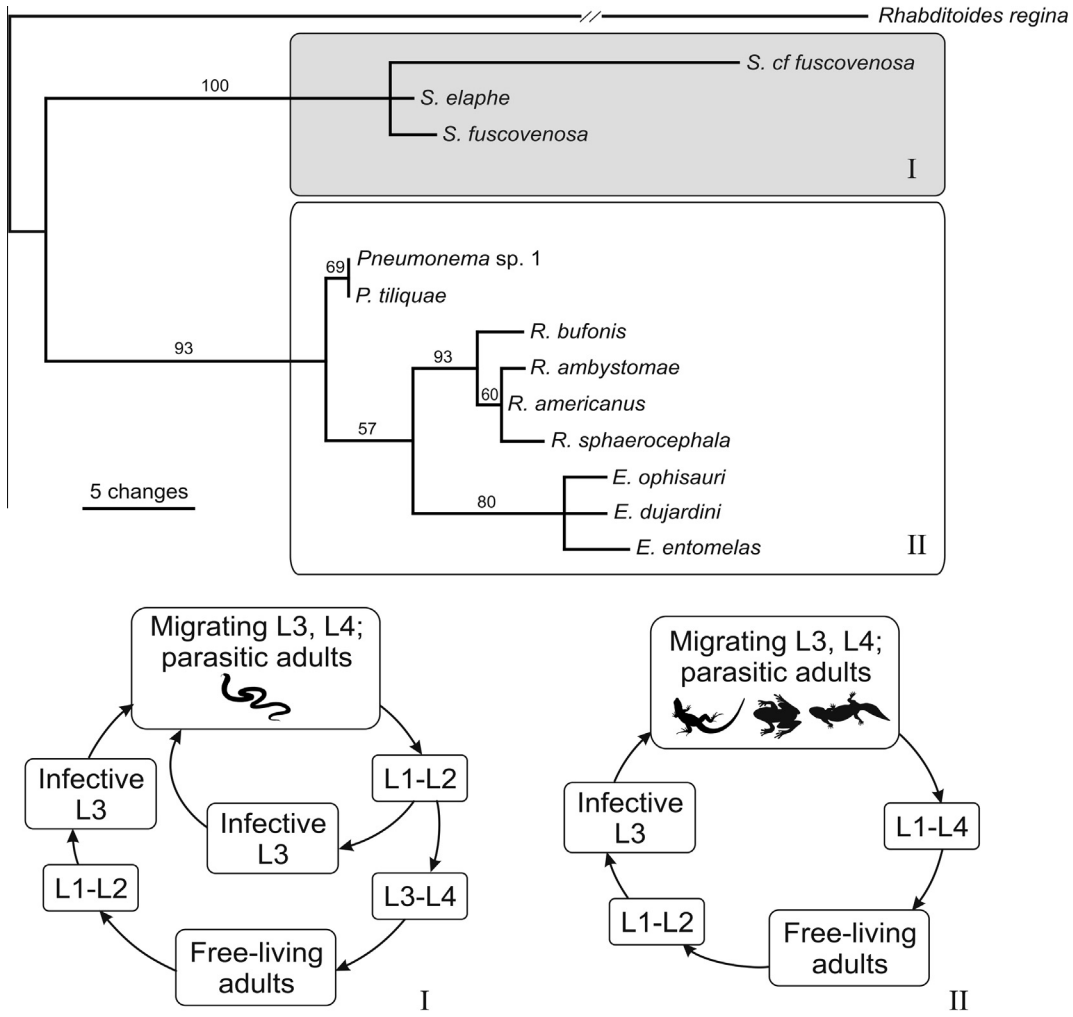

Fig. 1. Phylogenetic tree of the Rhabdiasidae with *Rhabditoides regina* as an outgroup, showing two main clades of the Rhabdiasidae and corresponding life cycles. Numbers above branches indicate posterior probabilities resulting from Bayesian analysis.

the Rhabidasidae and demonstrated that the three species from snakes (indicated as clade I on Fig. 1) formed a distinct sister group to the rest of the ingroup taxa. This result strongly supported the status of snake rhabdiasids as a separate genus and allowed us to use a species from snakes as the outgroup in the subsequent more detailed analysis of interrelationships among Rhabdiasidae.

Alignment II comprised a total of 1,668 sites (with alignment gaps) of which 1,484 sites aligned unambiguously. The taxon set included all available species of *Entomelas* and *Pneumonema* as well as several species of *Rhabdias* from various host groups and geographical regions to ensure representation of major *Rhabdias* lineages. This second analysis yielded a tree topology strongly supported by posterior probabilities (Fig. 2) that indicated monophyletic clades of *Entomelas* (100% support), *Pneumonema* (97%) and *Rhabdias* (100%). Among the five species comprising the *Entomelas* clade, two pairs of species, (*Entomelas ophisauri* (Kreis, 1939) + *Entomelas dujardini* (Maupas, 1916)) and (*Entomelas kazachstanica* Sharpilo & Vakker, 1972 + *Entomelas* sp.) were strongly supported at 95% and 100%, respectively. It is notable that the Eurasian *E. kazachstanica* was more closely related to an as-yet undescribed North American species, *Entomelas* sp., than to the three remaining *Entomelas* from the Palaearctic. Among the three species of *Pneumonema* (all from Australia) the only formally described species, *P. tiliquae*, clustered with the undescribed *Pneumonema* sp. 1. Although monophyly of genera was supported in this analysis, the interrelationship between genera remained ambiguous (posterior probability = 69 for the clade *Rhabdias* + *Pneumonema*).

The third phylogenetic analysis included *Pneumonema* as the outgroup and 25 available sequences of *Rhabdias*. Alignment III was 1,512 bp long, of which 1,500 sites could be aligned unambiguously. Although the monophyly of *Rhabdias* as a genus was strongly supported by our second analysis (Fig. 2), the internal interrelationships within the genus were not fully resolved. The resulting tree contained some polytomies and overall poorly resolved topology (Figs. 3 and 5). Nevertheless, some of the nodes were well supported. Among them is the cluster (*Rhabdias ambystomae* Kuzmin, Tkach & Snyder, 2001 + *R. sphaerocephala* + *Rhabdias americanus* Baker, 1979) demonstrating close relationships among species from European (*R. sphaerocephala*) and North American (*R. americanus*) toads and the parasite of North American salamanders (*R. ambystomae*). Another well-defined cluster includes *Rhabdias ranae* Walton, 1929, *R. joaquinensis* Ingles, 1935 and three forms temporarily identified as *R. joaquinensis*, although the latter taxa likely represent new species. All species in this cluster are parasitic in North American frogs.

The largest strongly supported clade in our third analysis unites a diverse group of species parasitic in amphibians in the Palaearctic, Oriental and Australian regions (Figs. 3 and 5). This is also the clade in which the majority of the internal topologies are well resolved and will be discussed below. One of the noteworthy sub-clades in this clade includes four species from Australia preliminarily identified as *Rhabdias hylae* Johnston et Simpson, 1942 by Dubey and Shine (2008). This topology also clearly shows that *R. bufonis* is most closely related to *R. hylae* from Australia and to species from southern and southeast Asia (*Rhabdias kongmonthaensis* Kuzmin, Tkach & Vaughan, 2005 and *R. bulbicauda*).

3.2. Erection of a new genus

Based on the results from the molecular data, peculiarities of the life cycles (see Anderson, 2000; Langford and Janovy, 2009, 2013; Kuzmin, 2013) and distinct morphological features we establish a new genus for rhabdiasid nematodes from snakes.

3.2.1. *Serpentirhabdias gen. nov.*

Rhabdiasidae. Small to medium-sized rhabdiasids. Body length 2–8 mm. Body widest at mid-length or somewhat anterior to it. Anterior end rounded, posterior end pointed. Body cuticle thin, somewhat thicker on anterior and posterior parts; cuticular inflations absent. Cuticular surface usually regularly transversely striated in anterior region. Oral opening round. Six distinct, circumoral lips, similar in size and shape, arranged in two lateral groups. Buccal capsule small, thin-walled, funnel-shaped, or cup-like, or absent. Oesophagus club-shaped, rounded at anterior end, with posterior bulb. Nerve ring usually situated at midlength of oesophagus or slightly posterior to it. Excretory glands distinct. Genital system amphidelphic, typical of the family. Seminal receptacles distinct, represented by thick-walled distal parts of oviducts. In most species eggs not numerous (about 100 in larger species), usually containing embryo in early cleavage stages. Vulva pre-equatorial or equatorial. Tail conical, with sharpened tip, often with needle-shaped cuticular ending.

Life cycles include combination of homogony and heterogony.

Parasitic in snakes (Reptilia: Squamata: Serpentes), most species in Colubridae.

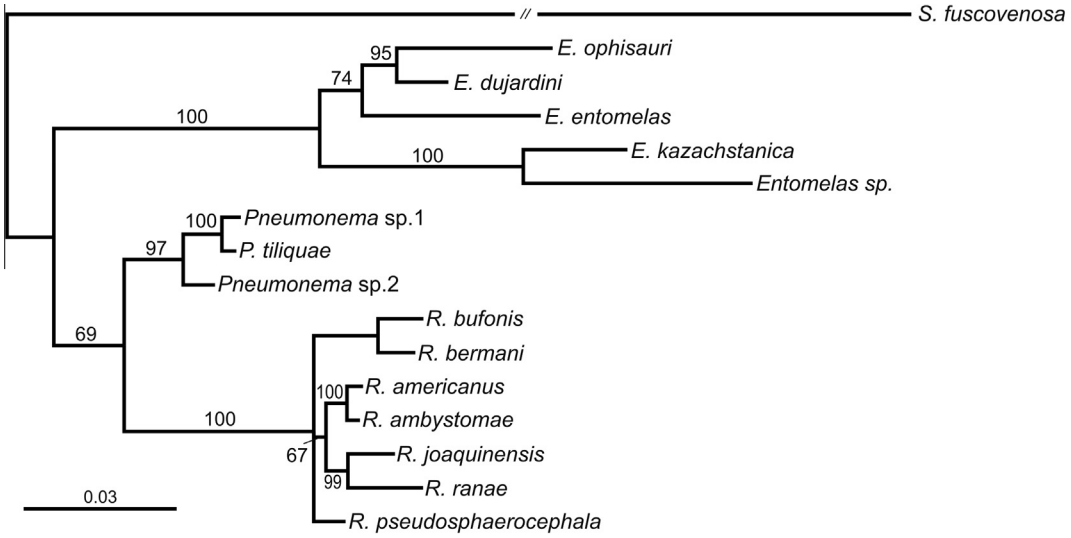

Fig. 2. Phylogenetic tree of *Rhabdias*, *Entomelas* and *Pneumonema* with *Serpentirhabdias fuscovenosa* as an outgroup. Numbers above branches indicate posterior probabilities resulting from Bayesian analysis.

Thirteen known species, distributed in the Holarctic, Neotropical, Oriental and Australian realms.

Type species: *Serpentirhabdias elaphe* (Sharpilo, 1976) comb. nov. (Syn.: *R. elaphe* Sharpilo, 1976).

3.2.2. Remarks

In its general morphology and its specificity to snakes, *Serpentirhabdias* gen. nov. resembles *Acanthorhabias* Pereira, 1927. The two genera differ in the morphology of the anterior end: *Serpentirhabdias* possesses six lips while *Acanthorhabdias* has eight hook-like projections (Pereira, 1927; Artigas et al., 1973). *Serpentirhabdias* is morphologically similar to *Rhabdias*, especially in having a small, thin-walled buccal capsule. In *Serpentirhabdias*, however, the body cuticle lacks prominent inflations with irregular folds characteristic of *Rhabdias*. *Serpentirhabdias* has six distinct lips of similar size arranged in two lateral groups. Some *Rhabdias* species (e.g. *R. bufonis*, *R. ambystomae*) also have six lips, but they are not arranged in lateral groups. In addition, in contrast to *Serpentirhabdias*, the lateral lips in *Rhabdias* are situated at a greater distance from the oral opening than the submedian ones. Gravid specimens of *Serpetirhabdias* spp. generally have fewer eggs in uteri than *Rhabdias* spp. and the eggs are usually at early cleavage stages when they are laid (Baker, 1978; Kuzmin, 2013). In contrast, in *Rhabdias* spp. most eggs in uteri contain fully developed larvae.

Some species of *Serpentirhabdias* (*Serpentirhabdias horigutii* (Yamaguti, 1943), *Serpentirhabdias pearsoni* (Kuzmin & Tkach, 2008, *Serpentirhabdias vibakari* (Kuzmin, 1999)) lack a buccal capsule which makes them similar to *Chabirenia* Lhermitte-Vallarino, Bain, Deharo, Bertani, Voza, Attout & Gaucher, 2005. The two genera also share a comparatively small body size. *Serpentirhabdias* differs from *Chabirenia* by the absence of longitudinal cuticular crests on the surface of the body and the absence of oesophageal onchia (teeth) in the buccal cavity. *Chabirenia cayennensis*, the only known species of the genus, is parasitic in lizards, *Ameiva ameiva* (Linnaeus, 1758), whereas all *Serpentirhabdias* spp. are parasites of snakes.

In the shape and size of the buccal capsule, *Serpentirhabdias* is also close to *Pneumonema*. However, *Serpentirhabdias* spp. lack the spines on the surface of the body characteristic of *Pneumonema*. *Pneumonema tiliquae*, as well as two undescribed species of this genus sequenced by us (Fig. 2), are specific to scincid lizards, whereas all *Serpentirhabdias* spp. are parasites of snakes.

The life cycles of five species of *Serpentirhabdias* are known and include both homogony and heterogony with one or the other mode predominating in different species (Chu, 1936b; Langford and Janovy, 2009; Kuzmin, 2013). Only heterogony has been reported in the life cycles of *Rhabdias*, *Entomelas* and *Pneumonema* (Seurat, 1920; Ballantyne, 1991; Langford and Janovy, 2009; Kuzmin, 2013) and only homogony has been observed in *Chabirenia* (Lhermitte-Vallarino et al., 2005).

4. Discussion

4.1. Taxonomy

Three commonly recognised genera of Rhabdiasidae, *Rhabdias*, *Entomelas* and *Pneumonema*, were included in the present analysis. The molecular phylogeny supports the status of *Entomelas* and *Pneumonema* as natural monophyletic taxa (Fig. 2). The analysis

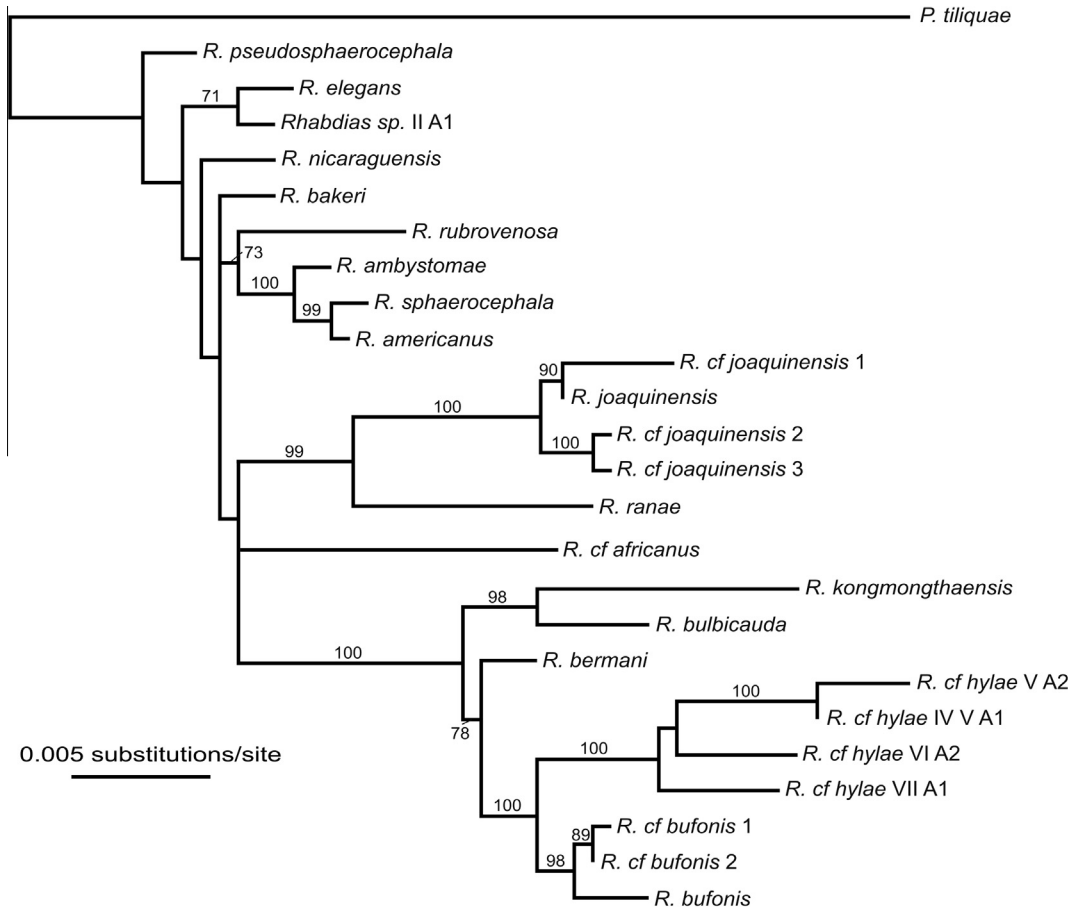

Fig. 3. Phylogenetic tree of *Rhabdias* with *Pneumonema tiliquae* as an outgroup. Numbers above branches indicate posterior probabilities resulting from Bayesian analysis.

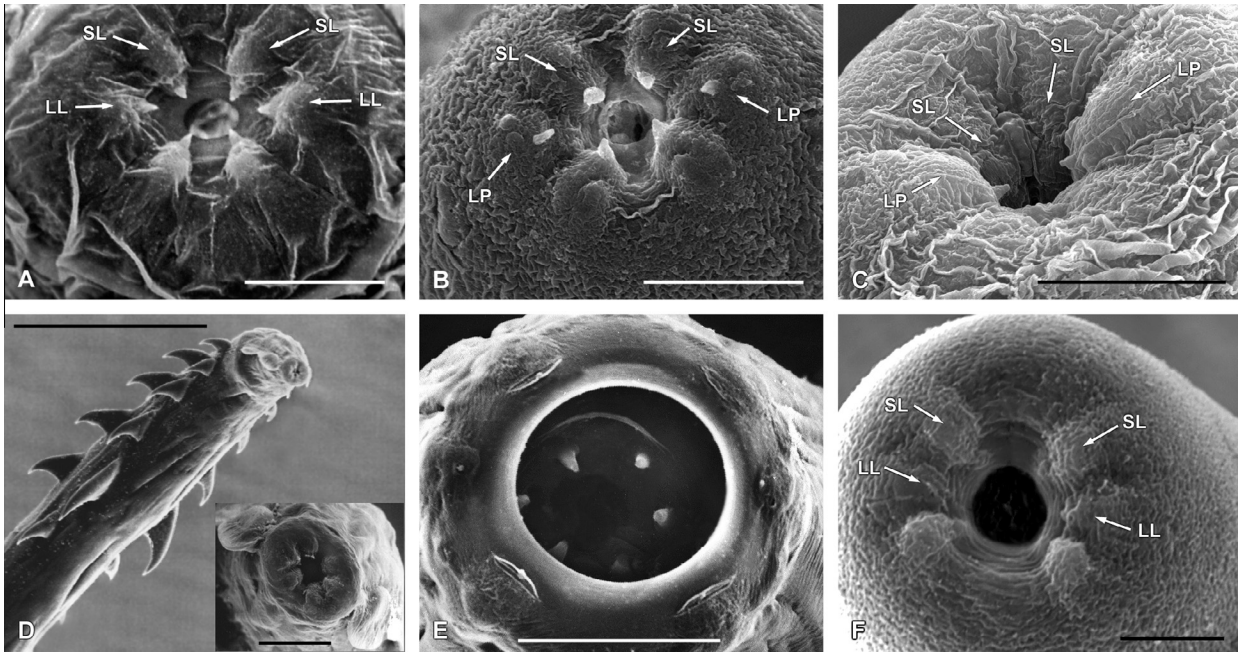

Fig. 4. Scanning electron microscopy of the representatives of main clades of Rhabdiasidae. (A) *Rhabdias ambystomae*, anterior end showing six circumoral lips of similar shape and size. (B) *Rhabdias ranae*, anterior end showing two lateral pseudolabia and four submedian lips. (C) *Rhabdias bakeri*, anterior end showing lateral pseudolabia and reduced submedian lips. (D) *Pneumonema tiliquae*, anterior part showing spines on the body surface and apical extremity (inset) showing cervical alae. (E) *Entomelas ophisauri*, anterior end showing circumoral lips and teeth in the buccal cavity. (F) *Serpentirhabdias eustreptos*, anterior end showing six lips in two lateral groups. LL, lateral lips; SL, submedian lips; LP, lateral pseudolabia. Scale bars: A–C and F = 10 μm; D = 200 μm, inset = 20 μm; E = 50 μm.

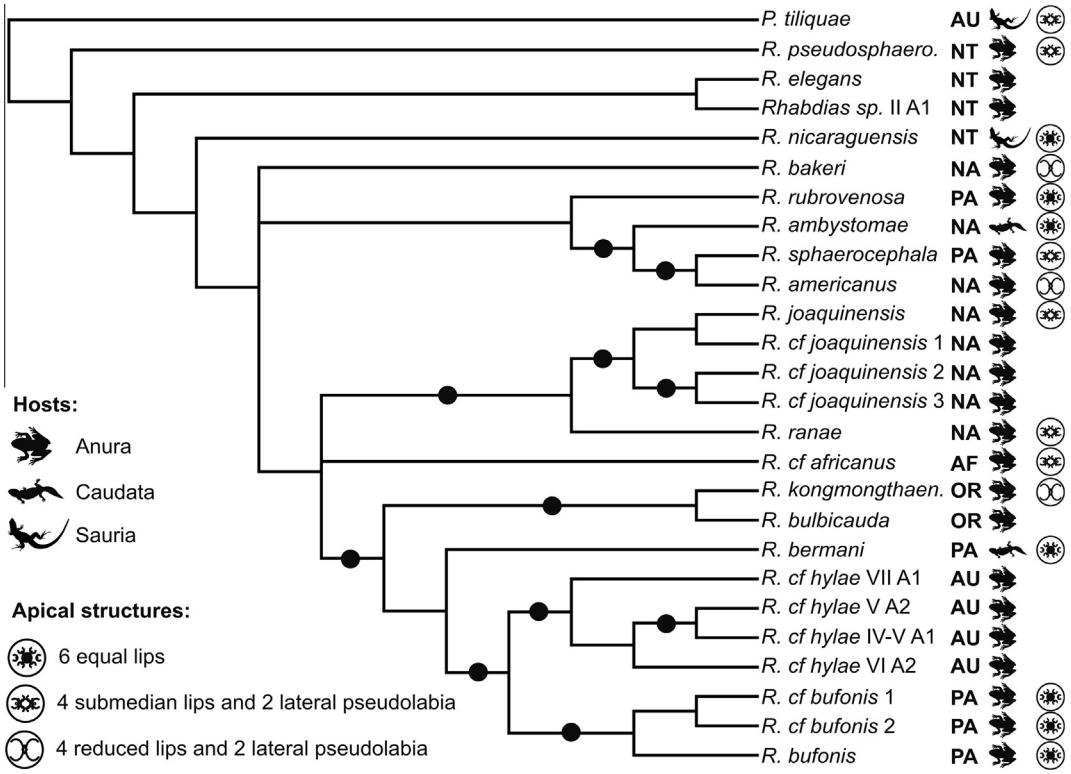

Fig. 5. Phylogenetic tree of *Rhabdias* species with *Pneumonema tiliquae* as an outgroup. Rectangular cladogram is used for convenience of mapping hosts, morphological characters and distribution. Solid circles indicate posterior probabilities of 95–100%. Branch support is based on posterior probabilities resulted from Bayesian analysis. Zoogeographical regions: AF, Afrotropical; AU, Australian; NA, Nearctic; NT, Neotropic; OR, Oriental; PA, Palaearctic.

revealed *Rhabdias* as a polyphyletic group consisting of two distinct clades, one of which, *Seprentirhabdias*, is a sister group to the remaining Rhabdiasidae (Fig. 1). Previously, Tkach et al. (Tkach, V., Kuzmin, Y., Snyder, S.D., 2008. Molecular insight into host specificity, life cycles and geographical distribution of the Rhabdiasidae (Nematoda). Xth European Multicolloquium Of Parasitology, Paris – France, SY06/04-02) concluded that *Rhabdias* from snakes should be allocated to an independent genus based on a molecular

phylogeny that included representatives of the same rhabdiasid genera as used in the present phylogeny; these were published in conference proceedings, thus no new genus was formally established. Later, Langford and Janovy (2013) came to a similar conclusion. However, due to the lack of any other rhabdiasids (other than *Rhabdias*) in their phylogeny, Langford and Janovy (2013) could not clearly demonstrate the separation of *Rhabdias* from species infecting snakes into a genus-level clade. In the present work, we have confirmed the conclusions made by Tkach et al. (2008) and Langford and Janovy (2013) and formally established the new genus *Serpentirhabdias* for this distinct lineage of rhabdiasids.

The species composition of the new genus cannot be determined definitively because of the absence of molecular and life cycle data for most rhabdiasids parasitic in snakes. Based on the available morphological and molecular data as well as information on life cycles, we can confidently include the following species in *Serpentirhabdias*: *S. elaphe* (Sharpilo, 1976), *S. fuscovenosa* s. l. (Railliet, 1899) comb. nov., *Serpentirhabdias eustreptos* (McCallum, 1921) comb. nov. and *Serpentirhabdias agkistrodonis* (Sharpilo, 1976) comb. nov. The following species are assigned to the new genus based only on their morphological similarity to the above group and their specificity to snakes: *Serpentirhabdias filicaudalis* (Barella, dos Santos & da Silva, 2009) comb. nov., *S. horigutii* (Yamaguti, 1943) comb. nov., *Serpentirhabdias kurilensis* (Sharpilo, 1976) comb. nov., *Serpentirhabdias labiata* (Pereira, 1927) comb. nov., *Serpentirhabdias lamothei* (Martinez-Salazar & Leon-Regagnon, 2007) comb. nov., *S. pearsoni* (Kuzmin & Tkach, 2008) comb. nov., *Serpentirhabdias vellardi* (Pereira, 1928) comb. nov., and *S. vibakari* (Kuzmin, 1996) comb. nov. *Serpentirhabdias martinoi* (Kurochkin and Guskov, 1963) comb. nov., an eye parasite of *Natrix natrix* (Linnaeus) (Serpentes: Colubridae) known from a single record (Kurochkin and Guskov, 1963), is included in the new genus provisionally until more data become available for this species.

Yamaguti (1943) erected the subgenus *Ophiorhabdias* Yamaguti, 1943 for the species *Rhabdias (Ophiorhabdias) horigutii*. The subgenus was supposed to include *Rhabdias* spp. parasitising snakes and was differentiated from the subgenus *Rhabdias* from amphibians based on the absence of the buccal capsule in *Ophiorhabdias* and its presence in *Rhabdias* (Yamaguti, 1943). However, Yamaguti's differentiation was erroneous because even at the time of his publication the buccal capsule was known in some species from snakes, e.g. *R. fuscovenosa* and *R. eustreptos*. Sharpilo (1976) elevated *Ophiorhabdias* to a genus including a single species, *O. horigutii,* while the rest of the species from snakes remained allocated to *Rhabdias*. Later, Baker (1980) synonymised *Ophiorhabdias* with *Rhabdias* considering the morphological differences of *O. horigutii* as insufficient for generic status.

Serpentirhabdias fuscovenosa has been reported from a number of snake species in both Eurasia and North America (Baker, 1987; Kuzmin et al., 2003; Kuzmin, 2013). The species was originally described from Europe (France) and is broadly distributed in Palaearctic. Our molecular comparison of *S. fuscovenosa* from North America (USA) and Europe (Ukraine) clearly indicates that these nematodes belong to two separate species-level genetic lineages. Although their morphological differentiation was beyond the scope of our study, it is clear that the Nearctic form needs to be described as a new species.

Some of the species of *Entomelas* used in the present study had been previously assigned to other genera. Sharpilo (1976) included *E. dujardini* and *E. kazachstanica* in *Paraentomelas* Sharpilo, 1976, and left *E. ophisauri* in *Hexadontophorus* Kreis, 1939, in which the species was originally placed. Baker (1980) synonymised all three above species with *Entomelas entomelas* (Dujardin, 1845). As a result, the genera *Paraentomelas* and *Hexadontophorus* were also synonymised with *Entomelas*. Our molecular data confirmed the validity of all four named *Entomelas* species used in our study. In the present molecular phylogenetic analysis, *E. dujardini* and *E. kazachstanica* appeared in different clades within *Entomelas* (Fig. 2) thus confirming that the genus *Paraentomelas* proposed by Sharpilo (1976) does not represent a monophyletic group. Conversely, *E. ophisauri* appears as sister species to *E. dujardini* (Fig. 2). Thus, the topology of the *Entomelas* clade confirms Baker's (1980) synonymy of *Paraentomelas* and *Hexadontophorus* with *Entomelas*.

4.2. Biology

The general topology of the molecular phylogenetic tree (Fig. 1) reflects differences in the life cycles of Rhabdiasidae. Homogony along with heterogony is present in the life cycles of species of *Serpentirhabdias*, while species from the remaining clades (*Rhabdias*, *Entomelas*, *Pneumonema*) possess heterogonic life cycles.

Entomelas entomelas differs from the majority of other rhabdiasids by localisation in the pharynx and oesophagus of its host, the anguid lizard *Anguis fragilis* Linnaeus, instead of in the lungs. In the molecular phylogenetic tree (Fig. 2) *E. entomelas* does not show a clear affinity to one of the two well-supported clades within *Entomelas*. Taking into account that the rest of the species of the genus are parasitic in the lungs, we hypothesise that *E. entomelas* has colonised the digestive tract secondarily. Data on the life cycle of *E. entomelas* indirectly support this hypothesis as immature specimens of the species were found in the host lungs from which they migrate to the pharynx (Kuzmin, 2013).

In the free-living generation, females of *Serpentirhabdias* have from 8 to 17 eggs in the uterus, whereas in most species of *Rhabdias*, free-living females have no more than four eggs (Pereira, 1927; Kloss, 1974; Baker, 1979; Lhermitte-Vallarino et al., 2008, 2009; Langford and Janovy, 2009; Kuzmin, 2013). These differences may represent an additional distinguishing character separating *Serpentirhabdias* and *Rhabdias*. It is notable that the egg number in the free-living females of *Entomelas* may vary significantly, ranging from three in *E. dujardini* and five in *E. entomelas*, to 16 in *E. ophisauri* and 18 in *E. kazachstanica* (Kuzmin, 2013). The former two species are parasitic in *A. fragilis* living in relatively humid habitats, while the latter two species parasitise the European legless lizard *Pseudopus apodus* (Pallas), also an anguid, but usually living in drier habitats. The phylogeny (Fig. 2) does not reflect the differences in fecundity of free-living females which seem to be the result of adaptations to different types of environment.

Among all nematodes with known life cycles that are currently included in the Rhabdiasidae, only *C. cayennensis* from the teiid lizard *Ameiva ameiva* lacks heterogony in its life cycle (Lhermitte-Vallarino et al., 2005). This unusual feature causes some doubts about the current systematic position of this genus. It is possible that *Chabirenia* belongs to one of the lineages of free-living rhabditoid nematodes that independently transitioned to parasitism in vertebrates. Future molecular phylogenetic studies should clarify this question which may also be of critical importance in understanding the evolution of parasitism among the Rhabdiasidae and related groups of nematodes.

4.3. Morphology

Four main monophyletic groups within the Rhabdiasidae (Figs. 1 and 2) represent three formerly recognised genera (*Rhabdias*, *Pneumonema*, *Entomelas*) and the new genus *Serpentirhabdias*. Considering the complex history of taxonomic re-shuffling and synonymies of rhabdiasids based solely or mostly on morphology, the molecular phylogeny provides an opportunity for re-assessment of the relative taxonomic values of characters used in rhabdiasid systematics.

Of all the characters mentioned in the diagnoses of *Rhabdias* by Travassos (1930) and Baker (1978) only the inflation of the body

cuticle, at least in some parts of the body, is shared by all species of the genus. Such inflation is formed by a thickened layer of mesocuticle (Bogoyavlenskiy, 1973) in adult *Rhabdias* after they enter the host lung and become gravid (Baker, 1979; Kuzmin, 2013). In most species, the inflation is more pronounced on the anterior and posterior portions of the body. However, cuticular inflation is not unique for *Rhabdias* and has also been observed in two *Entomelas* species, namely *E. dujardini* and *E. kazachstanica* (Sharpilo, 1976; Kuzmin, 2013). These two species of *Entomelas* do not form a monophyletic group within the clade of *Entomelas* (Fig. 2). Other rhabdiasid genera lack significant cuticular inflation, suggesting that this feature has evolved independently more than once in the evolutionary history of this group of nematodes. However, the presence of cuticular inflation appears to be synapomorphy within the genus *Rhabdias*.

According to our results, the distribution of morphological characters within *Rhabdias*, shows little consistency with the phylogenetic tree topology. For instance, the presence or absence of various structures (e.g., lips, pseudolabia) at the anterior end of *Rhabdias* as well as variations in their shape and position, have been widely used for species differentiation in this genus and have seemed to be promising for phylogenetic analysis. Baker (1978) separated *Rhabdias* species into three groups based on type and number of circumoral structures in gravid individuals (apical structures may be different in subadult specimens): species without lips, species with six lips, and species with two lateral pseudolabia. Further studies have demonstrated that Baker's (1978) suggestion that *R. bufonis* lacks lip was erroneous (Kuzmin, 2013) and two additional types of apical end morphologies have been revealed (Kuzmin et al., 2003, 2007; Kuzmin, 2013). Thus, five types of apical morphology in *Rhabdias* are currently known: (1) species without lips (*R. alabialis* Kuzmin, Tkach & Brooks, 2007), (2) species with six lips uniform in shape and size (e.g., *R. ambystomae*), (3) species with four normally developed submedian lips and two lateral pseudolabia (e.g., *R. ranae*, *R. joaquinensis*), (4) species with two lateral pseudolabia and four lips in the form of protuberances (e.g., *R. americanus*, *R. bakeri* Tkach, Kuzmin & Pulis, 2006), and (5) species with two pseudolabia and no lips (*R. bicornis* Lu, 1934; after Lu, 1934).

Anterior end morphology has been described in detail for a majority of species of *Rhabdias*, though not for all of the taxa used in the current study. Each of these species may be assigned to one of three groups: species with six lips, species with four normal lips and two pseudolabia, and species with two pseudolabia and four reduced lips (protuberances) (Fig. 4A–C). Mapping the apical structures on the phylogenetic tree has demonstrated a general lack of patterns consistent with the tree topology (Fig. 5). For instance, the clade containing *R. ambystomae* + *R. sphaerocephala* + *R. americanus* includes species having all three types of apical structures. At the same time, lateral pseudolabia are also present in species belonging to other clades within *Rhabdias*. *Rhabdias bakeri*, *R. americanus* and *Rhabdias kongmongthaensis* possess lateral pseudolabia and modified submedian lips but do not belong to the same monophyletic group. It seems that the formation of lateral pseudolabia is a general trend in the evolution of *Rhabdias* and similar structures might have appeared independently in several lineages within this large genus. Therefore, the presence or absence of pseudolabia as well as their number and shape are not reliable characters for grouping *Rhabdias* species as was suggested by Baker (1978). As demonstrated by the current study, similarity in anterior end morphology does not necessarily mean that the species are closely related and vice versa, species grouped in very strongly supported clades may have different apical structures (Fig. 5). Despite their limited utility for phylogenetic inference, the apical structures remain among the best characters for species differentiation in this group of nematodes.

Pneumonema tiliquae is the only formally described species of this genus but our data suggest existence of at least two additional species, one parasitic in the scincid lizard *Tiliqua scincoides* (White, 1790) and the other in the scincid *Cyclodomorphus gerrardii* Shea, 1990. The specimens of these putatively new *Pneumonema* were not suitable for overall morphological description but all specimens in the strongly supported *Pneumonema* clade (Fig. 2) are united by two unique synapomorphies, namely the presence of longitudinal rows of cuticular spines on the body surface and cervical alae (Fig. 4D).

The presence of six onchia (teeth) on the anterior edge of oesophagus is characteristic of species of *Entomelas* (Fig. 4E) and may be considered as a synapomorphic character for this clade. In two *Entomelas* species, namely *E. dujardini* and *E. kazachstanica*, three of the six teeth are reduced (Kuzmin, 2013) and are difficult to observe under the light microscope which prompted Sharpilo (1976) to conclude that they are absent in these two species. On the phylogenetic tree, *E. dujardini* and *E. kazachstanica* belong to different clades (Fig. 2) which suggests that the tendency to a partial reduction of teeth has evolved independently in these two species parasitic in two different anguid lizards.

Species of the clade *Serpentirhabdias* share the number and arrangement of apical structures. All species whose morphology of the apical end has been properly studied have six lips characteristically arranged in two lateral groups (Fig. 4F). A thin body cuticle is another character typical of this genus.

4.4. Geographical distribution

The distribution of Rhadiasidae is limited by the distribution of their amphibian and reptilian hosts. *Rhabdias* is the most widely distributed genus of the family with representatives of the genus known from all continents with the exception of Antarctica. The distribution of *Rhabdias* as a whole does not seem to follow any biogeographical pattern (Fig. 5). Some of the well-supported clades within the genus comprise species limited in distribution to either continents or large geographic regions within continents. One of these clades consists of species occurring in the Nearctic (*R. ambystomae*, *R. americanus*) and Western Palaearctic (*R. sphaerocephala*) (Fig. 5). Notably, Palaearctic and Nearctic species do not form separate sub-clades within this cluster. For instance, the North American species *R. americanus* is more closely related to the European *R. sphaerocephala* than to the other North American species in the same clade, *R. ambystomae*. These relationships indicate that the group evolved before the breakup of Laurasia in the Palaeocene and early Eocene (Irving, 2005; Pramuk et al., 2008).

Another clade consists of the species occurring in the southern and southeastern Asia (*R. kongmongthaensis*, *R. bulbicauda*), the eastern Palaearctic (*R. bermani*), Australia (*R.* cf *hylae*) or broadly distributed in the Palaearctic (*R. bufonis* and related forms). Although the derived position of the Australian/transpalaearctic clade in relation to Asian species is not sufficiently supported in our tree (only 78%) one may hypothesise that the Australian *Rhabdias* included in the present analysis may have evolved from Asian ancestors that arrived on the continent with the colonisation of Australia by Asian Microhylidae and Ranidae (anurans) (Tyler, 1989). Inclusion of additional *Rhabdias* species from Southeast Asia and Australasia in future analyses is necessary in order to test this hypothesis. Inadequate taxon sampling also inhibits our understanding of the origin and evolution of African and Neotropical species of *Rhabdias*.

Pneumonema is the only genus of rhabdiasids endemic to Australia, with species parasitising scincid lizards belonging to the closely related genera *Tiliqua* Gray, 1825 and *Cyclodomorphus* Fitzinger, 1843. Species of *Tiliqua* are distributed in New Guinea

and several other islands (Gorseman, 1998), raising the possibility that *Pneumonema* enjoys a wider distribution outside of Australia.

Species of *Entomelas* used in the present analysis occur in the western Palaearctic (*E. entomelas*, *E. dujardini*, *E. kazachstanica* and *E. ophisauri*) and southern Nearctic (*Entomelas* sp.). The Nearctic species is nested among the Palaearctic species (Fig. 2). Three additional species of *Entomelas* that were not available for the current study, are known from the Neotropics (Central America) (Martínez-Salazar and León-Règagnon, 2005; Bursey and Goldberg, 2006) and one species, *Entomelas cruszi*, occurs on Sri Lanka (Baker, 1980). These patterns of distribution are generally consistent with a Laurasian origin of the genus with subsequent colonisation of Sri Lanka.

Species of *Serpentirhabdias* are known from all zoogeographical realms except the Afrotropical. Our analysis included three species of *Serpentirhabdias* occurring in the western Palaearctic (*S. elaphe*, *S. fuscovenosa*) and Nearctic (*S.* cf *fuscovenosa*). More species of the genus need to be sequenced and analysed in order to explore the interrelationships among species of *Serpentirhabdias* and their geographical distribution.

4.5. Host associations

Species of Rhabdiasidae parasitise amphibians, lizards of the families Polychrotidae, Agamidae, Chamaeleonidae, Scincidae and Anguidae, and snakes from Colubridae and Viperidae (Sharpilo, 1976; Baker, 1987; Kuzmin, 2003; Lhermitte-Vallarino et al., 2010; Bursey et al., 2012). *Rhabdias* has the widest host range, parasitising amphibians from three orders and various families, as well as lizards from the families Polychrotidae, Agamidae and Chamaeleonidae. The present study examined species parasitising caudatan and anuran amphibians as well as one species from polychrotid lizards. The caudatan parasites *R. bermani* and *R. ambystomae* each belong to a separate well-supported clade (100%) within *Rhabdias* (Fig. 5). *Rhabdias bermani* is nested among parasites of anurans from various families occurring in Asia and Australia. *Rhabdias ambystomae* is closely related to species parasitising bufonids in North America and Europe. This tree topology suggests that colonisation of caudatan amphibians has occurred more than once in the evolutionary history of *Rhabdias*.

Among three strongly supported clades within *Rhabdias* (Figs. 3 and 5), only the clade *R. ranae* + *R.* cf *joaquinensis* is specific to a single host genus, namely *Lithobates* Fitzinger, 1843. The other two clades include parasites of different orders and families. For instance, species of the clade (*R. kongmongthaensis* + *R. bulbucauda*) + *R. bermani* + (*R.* cf *hylae* + *R.cf bufonis*) parasitise hosts from the families Rhacophoridae, Bufonidae, Hynobiidae (Caudata), Hylidae, and Ranidae, correspondingly. Since the clades in *Rhabdias* parasitising amphibians are defined geographically rather than by host taxa we presume that host switching and ecological fitting were evolutionarily more important than association with particular host taxa.

One species from polychrotid lizards, *R. nicaraguensis* Bursey, Goldberg & Vitt, 2007, used in the present analysis is related to species parasitic in amphibians, though its exact affinities are not resolved on the phylogenetic tree. Other *Rhabdias* species parasitising lizards are known from Polychrotidae in South America, Chamaeleonidae in Africa and Madagascar, and Agamidae in South-East Asia (Kuzmin, 2003; Bursey et al., 2003, 2007, 2012; Martínez-Salazar, 2006; Lhermitte-Vallarino et al., 2010; Tkach et al., 2011; Kuzmin et al., 2012). These host families belong to a monophyletic group, Infraorder Iguania, which has a Gondwanan origin (Hedges and Vidal, 2009). *Rhabdias* parasitising Iguania may compose a monophyletic group of the same origin. Alternatively, they may represent separate clades that originated independently on each continent.

Pneumonema is specific to the scincid lizard genera *Tiliqua* and *Cyclodomorphus*. Three additional rhabdiasid species known from the Scincidae, namely *Kurilonema markovi* Szczerbak & Sharpilo, 1969, *K. browni* Kuzmin and Tkach, 2011 and *Neoentomelas asatoi* Hasegawa, 1989, are morphologically distinct from *Pneumonema* (Sharpilo, 1976; Hasegawa, 1989; Kuzmin and Tkach, 2011). Future molecular studies are necessary to explore the interrelationships among the rhabdiasids of scincids.

Species of *Entomelas* are parasitic in lizards of the family Anguidae, except for *E. cruszi* Baker, 1980 from Agamidae (Baker, 1980; Martínez-Salazar and León-Règagnon, 2005; Bursey and Goldberg, 2006; Kuzmin, 2013). Five species of *Entomelas* in the present study are parasites of Anguinae: *Ophisaurus* sp. (*Entomelas* sp.), *A. fragilis* (*E. entomelas* and *E. dujardini*), *P. apodus* (*E. ophisauri* and *E. kazachstanica*) but the parasites of *A. fragilis* are not sister to one another in the current analysis, nor are the parasites of *P. apodus* (Fig. 2). Baker (1980) doubted the presence of two species of the same genus in one host and considered the different worms as different ontogenetic stages. Present molecular data demonstrate the validity of each *Entomelas* species but they do not explain the presence of pairs of species in the same host. A potential explanation lies in peculiarities of the site specificity of gravid worms of each species. In *A. fraglis*, *E. dujardini* inhabits lungs, and *E. entomelas* inhabits the pharynx and anterior part of oesophagus (Sharpilo, 1976). In *P. apodus*, both species inhabit lungs, but gravid individuals of *E. ophisauri* were commonly observed in the body cavity, especially in heavily infected lizards (Sharpilo, 1976; Kuzmin, 2013). Thus, in each host species there is one species of *Entomelas* with localisation in lungs, typical for Rhabdiasidae, and the other one reaching maturity elsewhere. It is possible that the ancestors of *E. entomelas* and *E. ophisauri* may once have parasitised different anguid species and were captured by *A. fragilis* and *P. apodus*. Since the lungs of the new hosts were already occupied with *E. dujardini* and *E. kazachstanica*, subadult *E. entomelas* acquired the ability to continue migration from lungs to the pharynx, and *E. ophisauri* acquired the ability to attain maturity in the body cavity.

Anguinae is a monophyletic group that is presumed to have originated in North America and entered Europe in Eocene through the North-Atlantic terrestrial bridge (Macey et al., 1999). The common origin of North-American and European Anguinae is in agrement with the monophyly of the clade *Entomelas*. Other species of the genus, *E. campbelli* Martinez-Salazar & Leon-Regagnon, 2005, *E. floresvillelai* Martinez-Salazar & Leon-Regagnon, 2005 and *E. duellmani* Bursey and Goldberg, 2006, are parasitic in anguids of the subfamily Gerrhonotinae (Martínez-Salazar and León-Règagnon, 2005; Bursey and Goldberg, 2006), a sister group to Anguinae (Macey et al., 1999). Inclusion of these species into future phylogenetic analyses will allow to further investigate the co-evolution between *Entomelas* and different anguid lineages.

Baker (1984) posited that Rhabdiasidae were initially parasitic in amphibians and colonised reptiles secondarily. According to the present phylogenetic analysis, Rhabdiasidae parasitic in reptiles do not belong to a monophyletic group but are represented in four distinct clades (Figs. 2 and 5). Thus it appears that at least three major host switching events might have occurred in the evolutionary history of the family which resulted in the formation of *Serpentirhabdias*, *Entomelas* and *Pneumonema*. Host switching also appears to be a hallmark of *Rhabdias* that included numerous exchanges between different lineages of anuran and caudatan amphibians as well as a transition to iguanian lizards. Sequencing of additional, more variable, genes in future will likely allow to achieve better resolution in this morphologically uniform genus and further examine the evolution of its host associations.

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References

Anderson, R.C., 2000. Nematode Parasites of Vertebrates: Their Development and Transmission. CABI Publishing, Wallingford, UK.

Artigas, P., Araujo, P., Graeiro, A., 1973. Redescrição de *Acanthorhabdias acanthorhabdias* Pereira, 1927 (Nematoda: Rhabditoidea). Arj. Inst. Biol. São Paulo 40, 33–37.

Baker, M.R., 1978. Morphology and taxonomy of *Rhabdias* spp. (Nematoda: Rhabdiasidae) from reptiles and amphibians of southern Ontario. Can. J. Zool. 56, 2127–2141.

Baker, M.R., 1979. The free-living and parasitic development of *Rhabdias* spp. (Nematoda: Rhabdiasidae) in amphibians. Can. J. Zool. 57, 161–178.

Baker, M.R., 1980. Revision of *Entomelas* Travassos, 1930 (Nematoda: Rhabdiasidae) with a review of genera in the family. Syst. Parasitol. 1, 83–90.

Baker, M.R., 1984. Nematode parasitism in amphibians and reptiles. Can. J. Zool. 62, 747–757.

Baker, M.R., 1987. Synopsis of the Nematoda parasitic in amphibians and reptiles. Mem. Univ. Newfoundland. Occas. Pap. Biol. 11, 1–325.

Ballantyne, R.J., 1991. Life history and development of *Pneumonema tiliquae* (Nematoda: Rhabdiasidae). Int. J. Parasitol. 21, 521–533.

Bogoyavlenskiy, Yu K., 1973. Structure and Function of Integumentary Tissues of Parasitic Nematodes. Nauka, Moscow.

Bursey, C.R., Goldberg, S.R., 2006. Helminths in *Mesaspis monticola* (Squamata: Anguidae) from Costa Rica, with the description of a new species of *Entomelas* (Nematoda: Rhabdiasidae) and a new species of *Skrjabinodon* (Nematoda: Pharyngodonidae). Parasite 13, 183–191.

Bursey, C.R., Goldberg, S.R., Telford Jr., S.R., 2003. *Rhabdias anolis* n. sp. (Nematoda: Rhabdiasidae) from the lizard, *Anolis frenatus* (Sauria: Polychrotidae), from Panama. J. Parasitol. 89, 113–117.

Bursey, C.R., Goldberg, S.R., Vitt, L.J., 2007. New species of *Rhabdias* (Nematoda: Rhabdiasidae) and other helminths from *Norops capito* (Sauria: Polychrotidae) from Nicaragua. J. Parasitol. 93, 129–131.

Bursey, C.R., Hoong, D.C., Goldberg, S.R., 2012. A New Species of *Rhabdias* (Nematoda: Rhabdiasidae) in *Calotes versicolor* (Squamata: Agamidae) from Singapore. J. Parasitol. 98, 149–151.

Chabaud, A.-G., Brygoo, E.-R., Petter, A.-G., 1961. Description et caractères biologiques de deux noveaux *Rhabdias* malgaches. Ann. Parasitol. Hum. Comp. 36, 752–763.

Chu, T., 1936a. A review of the status of the reptilian nematodes of the genus *Rhabdias* with a description of *Rhabdias fuscovenosa* var. *catanensis* (Rizzo, 1902) new rank. J. Parasitol. 22, 130–139.

Chu, T., 1936b. Studies on the life history of *Rhabdias fuscovenosa* var. *catanensis* (Rizzo, 1902). J. Parasitol. 22, 140–160.

Cipriani, P., Mattiucci, S., Paoletti, M., Santoro, M., Nascetti, G., 2012. *Rhabdias esculentarum* n. sp. (Nematoda: Rhabdiasidae) from green frogs of the *Rana esculenta* species complex in Italy: molecular evidence, morphological description and genetic differentiation from its congeners in frogs and toads. Syst. Parasitol. 82, 131–146.

Dare, O.K., Nadler, S.A., Forbes, M.R., 2008. Nematode lungworms of two species of anuran amphibians: evidence for co-adaptation. Int. J. Parasitol. 38, 1729–1736.

Dubey, S., Shine, R., 2008. Origin of the parasites of an invading species, the Australian cane toad (*Bufo marinus*): are the lungworms Australian or American? Mol. Ecol. 17, 4418–4424.

Gorseman, P.D., 1998. *Tiliqua gigas*, de Nieuw-Guinea-blauwtongskink. Lacerta 57, 54–63.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hasegawa, H., 1989. *Neoentomelas asatoi* gen. et sp. n. (Nematoda: Rhabdiasidae) from skinks of the Ryukyu Archipelago, Japan. Proc. Helminthol. Soc. Wash. 56, 145–150.

Hedges, S.B., Vidal, N., 2009. Lizards, snakes, and amphisbaenians (Squamata). In: Hedges, S.B., Kumar, S. (Eds.), The Timetree of Life. Oxford University Press, pp. 383–389.

Irving, E., 2005. The role of latitude in mobilism debates. PNAS 102, 1821–1828.

Junker, K., Lhermitte-Vallarino, N., Barbuto, M., Ineich, I., Wanji, S., Bain, O., 2010. New species of *Rhabdias* (Nematoda: Rhabdiasidae) from Afrotropical anurans, including molecular evidence and notes on biology. Folia Parasitol. 57, 47–61.

Kloss, G.R., 1974. *Rhabdias* (Nematoda, Rhabditoidea) from the marinus group of *Bufo*. A study of sibling species. Arq. Zool. (São Paulo) 25, 61–120.

Kurochkin, Yu V., Guskov, E.P., 1963. New nematode species from the eyes of grass-snake. Helminth of human, animals and plants and their control. Izdatelstvo Akademii Nauk, Moscow, pp. 183–185 (In Russian).

Kuzmin, Y.I., 1997. The life cycle and the new data on distribution of *Rhabdias sphaerocephala* (Nematoda, Rhabdiasidae). Vestnik Zoologii 31, 49–57 (In Russian).

Kuzmin, Y.I., 1999. *Rhabdias agkistrodonis* (Nematoda: Rhabdiasidae): morphology, distribution and specificity. Folia Parasitol. 46, 59–66.

Kuzmin, Y., 2003. *Rhabdias japalurae* sp. nov. (Nematoda, Rhabdiasidae) from the japalures (Reptilia, Agamidae) and some notes on other *Rhabdias* spp. from lizards. Acta Parasitol. 48, 6–11.

Kuzmin, Y., 2013. Review of Rhabdiasidae (Nematoda) from the Holarctic. Zootaxa 3639, 1–76.

Kuzmin, Y., Miskov, R., 1999. The life cycle of *Rhabdias elaphe* Sharpilo, 1976 (Nematoda: Rhabdiasidae). Acta Parasitol. 44, 119–124.

Kuzmin, Y., Tkach, V.V., 2011. Description of a new species of *Kurilonema* (Nematoda: Rhabdiasidae) from lungs of the skink *Sphenomorphus abdictus aquilonius* (Reptilia: Squamata: Scincidae) in the Philippines. J. Parasitol. 97, 506–512.

Kuzmin, Y., Tkach, V.V., Snyder, S.D., 2003. The nematode genus *Rhabdias* (Nematoda: Rhabdiasidae) from amphibians and reptiles of the Nearctic. Comp. Parasitol. 70, 101–114.

Kuzmin, Y., Tkach, V.V., Brooks, D.R., 2007. *Rhabdias alabialis* sp. nov. and *R. pseudosphaerocephala* sp. nov. (Nematoda: Rhabdiasidae) in the marine toad, Bufo marinus (L.) (Lissamphibia: Anura: Bufonidae) in Central America. J. Parasitol. 93, 159–165.

Kuzmin, Y., Tkach, V.V., Bush, S.E., 2012. A new *Rhabdias* species (Nematoda: Rhabdiasidae) from agamid lizards in Luzon Island, Philippines. J. Parasitol. 98, 608–611.

Langford, G.J., Janovy Jr., J., 2009. Comparative life cycles and life histories of North American *Rhabdias* spp. (Nematoda: Rhabdiasidae): lungworms from snakes and anurans. J. Parasitol. 95, 1145–1155.

Langford, G.J., Janovy Jr., J., 2013. Host specificity of North American *Rhabdias* spp. (Nematoda: Rhabdiasidae): combining field data and experimental infections with a molecular phylogeny. J. Parasitol. 99, 277–286.

Leuckart, K.G.F.R., 1865. Zur Entwickelungsgeschichte des *Ascaris nigrovenosa*. Zugleich eine Erwiderung gegen Herrn Candidat Mecznikow. Archiv für Anatomie, Physiologie und Wissenschaftliche Medizin, Berlin, pp. 641–658.

Lhermitte-Vallarino, N., Bain, O., 2004. Morphological and biological study of *Rhabdias* spp. (Nematoda) from African chameleons with description of a new species. Parasite 11, 15–31.

Lhermitte-Vallarino, N., Bain, O., Deharo, E., Bertani, S., Voza1, T., Attout, T., Gaucher, P., 2005. A new rhabdiasid nematode, *Chabirenia cayennensis* n. g., n. sp., parasitic in the glands of the buccal mucosa of a South American saurian. Syst. Parasitol. 62, 151–160.

Lhermitte-Vallarino, N., Barbuto, M., Ineich, I., Wanji, S., Lebreton, M., Chirio, L., Bain, O., 2008. First report of *Rhabdias* (Nematoda: Rhabdiasoidea) from lungs of montane chameleons in Cameroon: description of two new species and notes on biology. Parasite 15, 553–564.

Lhermitte-Vallarino, N., Barbuto, M., Junker, K., Boistel, R., Ineich, I., Wanji, S., Bain, O., 2009. *Rhabdias rhampholeonis* n. sp. and *R. mariauxi* n. sp. (Nematoda, Rhabdiasoidea), first lung worms from leaf chameleons: description, molecular evidence and notes on biology. Parasitol. Int. 58, 375–383.

Lhermitte-Vallarino, N., Barbuto, M., Junker, K., Boistel, R., Bain, O., 2010. *Rhabdias* (Nematoda: Rhabdiasidae) from Chamaeleonidae (Sauria): two new species from *Trioceros ellioti* in east Africa and one from *Brookesia superciliaris* in Madagascar. Parasite 17, 91–105.

Lu, S.C., 1934. On *Rhabdias*, a genus of parasitic nematoda of Nanking. Sinensia 5, 164–172.

Macey, J.R., Schulte II, J.A., Larson, A., Tuniyev, B.S., Orlov, N., Papenfuss, T.J., 1999. Molecular phylogenetics, tRNA evolution, and historical biogeography in anguid lizards and related taxonomic families. Mol. Phylogenet. Evol. 12, 250–272.

Martínez-Salazar, E.A., 2006. A new rhabdiasid species from *Norops megapholidotus* (Sauria: Polychrotidae) from Mexico. J. Parasitol. 92, 1325–1329.

Martínez-Salazar, E.A., León-Règagnon, V., 2005. Two new species of *Entomelas* (Nematoda: Rhabdiasidae), parasites of *Barisia* spp. and *Mesaspis* spp. (Reptilia: Sauria) in Mexico. Zootaxa 958, 1–12.

Metchnikoff, I., 1865. Ueber die Entwicklung von *Ascaris nigrovenosa*. Archiv für Anatomie, Physiologie und Wissenschaftliche Medizin, Leipzig, pp. 409–420.

Pereira, C., 1927. Fauna helminthologica de ophidios brasileiros. Boletim Biologico, São Paulo 10, 179–185.

Pramuk, J.B., Robertson, T., Sites Jr., J.W., Noonan, B.P., 2008. Around the world in 10 million years: biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). Glob. Ecol. Biogeogr. 17, 72–83.

Schrank, F. von Paula, 1788. Verzeichniss der bischer hinlanglich bekannten Eingeweidewürmer. Munchen, Strobl.

Seurat, L.G., 1920. Histoire naturelle des nématodes de la Bérbérie. Première partie. Morphologie, développement, éthologie et affinités des nématodes. Université d'Alger, Faculté des Sciences, Fondation Joseph Azoubib. Travaux du Laboratoire de Zoologie Générale. S. Stamel, Algiers, Algeria.

Sharpilo, V.P., 1976. Parasitic Worms of Reptilians of the Fauna of the USSR. Naukova Dumka, Kiev.

Stiles, Ch.W., Hassall, A., 1905. The determination of generic types, and a list of roundworm genera, with their original and type species. Bureau of Animal Industry, U.S. Department of Agriculture, Bull. 79, Washington, Government Printing Office, USA.

Tkach, V., Pawlowski, J., 1999. A new method of DNA extraction from the ethanol-fixed parasitic worms. Acta Parasitol. 44, 147–148.

Tkach, V.V., Kuzmin, Y., Pulis, E.E., 2006. *Rhabdias bakeri* sp. n. from lungs of wood frog, *Rana sylvatica*, in North America: the last sibling of *Rhabdias ranae*? J. Parasitol. 92, 631–636.

Tkach, V.V., Kuzmin, Y., Brown, R.M., 2011. *Rhabdias mcguirei* sp. nov. (Nematoda: Rhabdiasidae) from the flying lizard, *Draco spilopterus*, (Squamata: Agamidae) of the northern Philippines. Acta Parasitol. 56, 406–411.

Travassos, L., 1930. Pesquizas helminthologicas realisados em Hamburgo. VII. Notas sobre os Rhabdiasoidea Railliet, 1916 (Nematoda). Mem. Inst. Oswaldo Cruz 24, 161–181.

Tyler, M.J., 1989. Australian Frogs. Viking O'Neil, Melbourne.

Yamaguti, S., 1943. *Rhabdias* (*Ophiorhabdias*) *horigutii* n. subg., n. sp. (Nematoda) from the lung of a Japanese snake *Natrix tigrina*. Annot. Zool. Jpn. 22, 8–10.