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HOST SPECIFICITY OF NORTH AMERICAN *RHABDIAS* SPP. (NEMATODA: RHABDIASIDAE): COMBINING FIELD DATA AND EXPERIMENTAL INFECTIONS WITH A MOLECULAR PHYLOGENY

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ABSTRACT: Lungworms of the cosmopolitan genus *Rhabdias* are among the most common parasites of amphibians and squamate reptiles. The present study used experimental infections, field studies, and a molecular phylogeny to determine the host specificity of 6 *Rhabdias* spp. that infect snakes and anurans from North America. The molecular phylogeny suggests *Rhabdias ranae* from Nebraska and Mississippi may represent separate, cryptic species. In addition, the phylogeny strongly supports separate clades for anuran and snake lungworms. Field studies and experimental infections indicate that snake lungworms are generalist snake parasites; however, laboratory experiments also suggest that lizards can be infected under some environmental conditions. Lungworms from anurans were found not to infect salamanders or reptiles, in nature or in the laboratory; anuran lungworm species ranged from strict host specificity, e.g., *R. ranae* from Nebraska, to relative generalist, e.g., *Rhabdias joaquinensis* from Nebraska. Overall, host specificity for species of *Rhabdias* does not provide support for the evolution of progressive specialization over time. For most species of lungworms, host specificity in nature appears to be limited by both ecological and physiological factors, which vary between species and their hosts. Furthermore, some lungworms, e.g., *Rhabdias bakeri* from Missouri, appear to be tracking host resources instead of host phylogenies, an example of ecological fitting.

Host specificity, defined as the number of host species a parasite can infect, is a universal and fundamental property of parasites, and knowledge of host range is vital to understanding parasite evolution and ecology. Host specificity provides insight into several intrinsic aspects of parasitism, such as parasite population dynamics, geographic limitations, taxonomic determinations, and past and future evolutionary avenues (Brooks and McLennan, 2002; Poulin, 2007). However, despite its importance, little information is available on the limitations of host range for most parasite species (Perlman and Jaenike, 2003), and, when available, host range is often inferred from field-collected data, i.e., realized host specificity, which likely represents only a fraction of the fundamental specificity that exists (Detwiler and Minchella, 2009). Experimental approaches provide a method to determine the fundamental host range by avoiding constraints inherent to the sampling of parasite communities in nature; e.g., experimentation provides assurance of host-parasite encounters (Snyder and Janovy, 1994; Janovy, 2002; Krasnov et al., 2004; Bolek and Janovy, 2007; Dare et al., 2008; Detwiler and Janovy, 2008). Thus, when fundamental and realized approaches to host specificity are combined it is possible to identify compatibility differences or preferences for particular host species (Detwiler and Minchella, 2009).

In nature, host range can be restricted by phylogenetic, ecological, physiological, and immunological parameters (Combes, 2001; Poulin, 2007). The effects of each parameter on host specificity are difficult to isolate, especially without an underlying evolutionary framework. However, when data on fundamental and realized host specificity are combined with a phylogeny, much insight into the constraints placed on parasite range by each of the 4 parameters can be obtained. A few recent studies have used this powerful methodology to successfully elucidate host specificity and provide insight into preferences for specific host species (Criscione et al., 2006; Edwards and Vidrine, 2006; Little et al., 2006; Munoz et al., 2007; Dare et al., 2008; Detwiler and Janovy, 2008; Bolek et al., 2009; Pizzatto and Shine, 2011).

The increased availability of molecular tools to study host-parasite co-evolution has been a key component in recent host specificity research. Knowledge of a group's evolutionary history can provide support for the hypothesis of progressive specialization in host-parasite lineages (Brooks and McLennan, 2003). Furthermore, host specificity based on co-speciation events is detectable only using phylogenetic methods (Adamson and Caira, 1994), and often the resulting specialization produces strict host specificity. Alternatively, a lack of congruent phylogenetic patterns suggests that current host range is not a result of common ancestry. If evolutionary history is not directly responsible for host range, then alternative factors, such as host-parasite ecological interactions, should be suspected. In this case, the phylogeny provides evolutionary context for exploring alternative factors that may influence host range (Poulin, 2007). Phylogenies are also useful when working with morphologically cryptic species, which have the potential to artificially alter measurements of host range (Jousson et al., 2000; Criscione et al., 2005; Emelianov, 2007). Cryptic species are often impossible to distinguish morphologically, yet their life histories and ecological interactions may vary widely, thus leading to substantial differences in host specificity.

Our previous study (Langford and Janovy, 2009) established the life histories and life cycles of 6 *Rhabdias* spp. from North American snakes and anurans, but little is known concerning their host specificity. Of the more than 70 species of *Rhabdias* worldwide only 4 species have been experimentally tested for fundamental host range. Chu (1936) exposed *Rhabdias fuscovenosa* obtained from a snake (*Nerodia sipedon* or *Thamnophis sirtalis*) to 2 species of frogs, a toad, and a turtle; he was unable to establish infections in these hosts and suggested *R. fuscovenosa* was restricted to snakes. Subsequently, Baker (1979) conducted cross-transmission experiments on *Rhabdias americanus*, *R. ranae* (herein assumed to be *Rhabdias bakeri*; see Tkach et al., 2006), and *R. fuscovenosa*. In agreement with Chu, he was unable to establish non-snake infections using *R. fuscovenosa*. However, despite an apparent lack of cross-transmission in nature, Baker was able to establish infections of *R. americanus*, obtained from

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Bufo americanus, in *Rana blairi*. He also established *R. bakeri* infections, obtained from *Rana sylvatica*, in *Bufo americanus*. Dare et al. (2008) successfully established *R. ranae*, collected from *Rana pipiens*, infections in sympatric *Rana blairi* metamorphs. However, molecular data suggested wild *Rana blairi* were not infected with *R. ranae*. Recently, Pizzatto and Shine (2011) found that *Rhabdias pseudospaerocephala* from the invasive cane toad, *Bufo* (*Rhinella*) *marinus*, was able to switch hosts and infect 2 species of native tree frogs (*Litoria* spp.). These 4 studies suggest that additional evolutionary avenues, i.e., opportunities, are available to *Rhabdias* spp. in nature, but such infections, if they occur, likely remain undetected due to small host sample sizes and spatiotemporal limitations in sampling.

Currently, amphibian *Rhabdias* spp. are considered specialists, i.e., host specific, whereas species of lungworms infecting snakes are considered generalists (Kuzmin 2001; Kuzmin et al. 2003; Tkach et al., 2006; Martínez-Salazar and León-Règagnon, 2007; Dare et al., 2008). This conclusion is based on field surveys and/or molecular sequences, but experimental infections and a molecular phylogeny are lacking. To address issues associated with host specificity, the present study's goals are to (1) determine both the realized and fundamental host specificity of 6 North American lungworm species from native amphibians and reptiles using experimental infections and field collections; (2) develop a molecular phylogeny to elucidate relationships between lungworms and reveal evolutionary and ecological avenues for, and constraints on, lungworm transmission and specificity; and (3) use the phylogeny to address an unanswered question from Langford and Janovy (2009). Our previous study found anuran and snake lungworms had distinctly different life cycles and suggested these differences could be reflected in the evolutionary history of these lungworms. If snake and anuran lungworms form separate clades, then the suggestions of Langford and Janovy (2009) would be supported.

MATERIALS AND METHODS

Amphibian and reptile field studies

Amphibians and reptiles were captured both day and night using hand capture, pit-fall traps, funnel traps, and drift fences and by cruising roads for dead and live animals (Heyer et al., 1994). Animals were transported to either the parasitology laboratory at the University of Nebraska–Lincoln's Cedar Point Biological Station in western Nebraska, or the vertebrate museum at the University of South Alabama, where they were killed and measured for snout-vent length (SVL). All organs and tissues were examined for *Rhabdias* spp. within 24 hr of collection. Some lungworms were removed and fixed in hot 70% ethyl alcohol, then cleared in glycerol for identification. Most adult hermaphrodites were identified alive, then set aside for use in host specificity experiments. In all experiments and collections, hosts were considered infected when gravid worms were collected from the lungs. Juvenile worms recovered from the body cavity were recorded, but not reported because there was no evidence these worms would reproduce. To ensure worms were provided sufficient time to establish in the lungs, all exposures were guided by the time periods provided by Langford and Janovy (2009). Representative specimens were cleared and temporarily mounted in glycerol for identification (Pritchard and Kruse, 1982). All lungworms were identified according to Kuzmin et al. (2003), supplemented by Tkach et al. (2006). Lungworm specimens were deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML 63512–63517).

During June and July 2006, the following anurans were collected from Lancaster County, Nebraska (40°51′N, 96°53′W): *Acris blanchardi* Harper, 1947, *Bufo* (*Anaxyrus*) *woodhousii* (Girard, 1854), *Hyla chrysoscelis* Cope, 1880, *Pseudacris triseriata* (Wied-Neuwied, 1838), *Rana* (*Lithobates*) *blairi* (Mecham, Littlejohn, Oldham, Brown, and Brown, 1973), *Rana* (*Lithobates*) *catesbeiana* (Shaw, 1802), and *Spea bombifrons* (Cope, 1863). During the same period, the following herpetofauna were collected from Keith County, Nebraska (41°18′N, 101°55′W): *Ambystoma tigrinum* (Green, 1825), *Aspidoscelis sexlineata* (Linnaeus, 1776), *Sceloporus undulatus* (Bosc and Daudin in Sonnini and Latreille, 1801), *Thamnophis sirtalis* (Linnaeus, 1758), and *Thamnophis radix* (Linnaeus, 1766). The following herp species were collected from Shawnee County, Kansas (39°02′N, 95°34′W): *A. blanchardi, B. woodhousii, H. chrysoscelis*, and *Pantherophis alleghaniensis* (Holbrook, 1836). In July 2007, the following anurans were collected from Saunders County, Nebraska (41°01′N, 96°17′W): *Bufo* (*Anaxyrus*) *americanus* (Holbrook, 1836), *B. woodhousii*, *H. chrysoscelis*, *P. triseriata*, *R. blairi*, and *R. catesbeiana*. In August 2007, the following herpetofauna were collected from the Grand Bay National Estuarine Research Reserve, Jackson County, Mississippi (30°25′N, 88°25′W): *Acris gryllus* (LeConte, 1825), *Agkistrodon piscivorous*, *Bufo* (*Anaxyrus*) *fowleri* (Hinckley, 1882), *Bufo* (*Anaxyrus*) *quercicus* (Holbrook, 1840), *Bufo* (*Anaxyrus*) *terrestris* (Bonnaterre, 1789), *Coluber constrictor* Linnaeus, 1758, *Gastrophryne carolinensis* (Holbrook, 1836), *Hyla cineria* (Schneider, 1799), *Hyla versicolor* LeConte, 1825, *Kinosternon subrubrum* (Lacépède, 1788), *Lampropelttis getula* (Linnaeus, 1766), *Nerodia fasciata* (Linnaeus, 1766), *Rana* (*Lithobates*) *clamitans* (Latreille, 1801), *Rana* (*Lithobates*) *sphenocephala* (Cope, 1886), *Scaphiophus hoolbrooki* (Harlan, 1835), *Thamnophis sauritus* (Linnaeus, 1766) , and *Trachemys scripta* (Schoepff, 1792). Finally, in April 2008, road-killed *P. triseriata, Rana sphenocephala*, and *Rana* (*Lithobates*) *sylvatica* (LeConte, 1825) were collected from Jefferson County, Missouri (38°17′N, 90°26′W). Numbers of hosts collected at each location are provided in Table I. Of note, not all of the individual hosts listed above are reported in Table I because some animals were used in experimental infections.

Acquiring uninfected hosts

Hosts used for experimental infections were collected during field surveys (see above). Young-of-the-year (YOY) amphibians were collected as eggs, tadpoles, metamorphs, or bred from captive adults or road-killed specimens, via artificial insemination of eggs, for experimental infections. Metamorphs were held for 2–3 wk, dependent on species, to allow prepatent infections to develop; infected individuals were not used in experimental infections. Given the young age of these uninfected metamorphs, we assumed they had not been infected in their lifetime, based on lungworm life history data provided by Langford and Janovy (2009).

Reptiles were not differentiated by size because they do not undergo metamorphosis and their immune system does not shift drastically during their lifetime (but see Ujvari and Madsen, 2006), as with amphibians (Rollins-Smith, 1998). Nevertheless, to alleviate unknown effects of age on infection parameters, no reptiles were exposed to worms before 3 wk of age. Captured reptiles were held for 3 wk to allow prepatent infections to develop, thus assuring only uninfected individuals were used in experiments. Uninfected snakes also were acquired by isolating wild, gravid individuals in the laboratory and housing offspring in cages that prevented transmission. The infection history of wild captured hosts, both amphibian and reptile, is unknown, but it seems reasonable to assume that some adults were previously infected with at least 1 species of lungworm. It is not known if host immune response is altered by prior lungworm infection.

Experimental lungworm infections

Free-living lungworms were acquired directly from parasitic adults removed from host lungs to ensure lungworm identification. Infective juveniles were raised in Petri dishes according to Langford and Janovy (2009). Hosts were exposed to infective lungworms according to Langford and Janovy (2009). In brief, this method involved orally pipetting infective lungworms obtained from snake hosts, which infect hosts only orally, into experimental hosts, whereas amphibian lungworms, which penetrate host dermal tissues, were placed in appropriately sized containers with experimental hosts. Hosts were each exposed to 30 infective juveniles, either per os or per container. The number of juvenile worms that failed to penetrate during exposures in containers was noted. Depending on cannibalistic tendencies, hosts were housed and cared for individually or in small groups. Cages were lined with absorbent paper towels and cleaned 2–3 times per wk to prevent development of infective juveniles in host feces and potential transmission. Snakes were fed frozen, thawed white fish (*Tilapia* sp.), and/or mice; anurans were fed a diet of commercial crickets (Top Hat Cricket Farm, Kalamazoo, Michigan) and cultured mealworms

TABLE I. Prevalence (P) and mean abundance (MA) ± standard deviation (SD) of *Rhabdias* spp. from anurans and snakes collected in Kansas (KS), Mississippi (MS), Missouri (MO), and Nebraska (NE).

	R. americanus		*R.bakeri*		*R. eustreptos*		*R. fuscovenosa*		*R. joaquinensis*		*R. ranae*	
Host species (location/n)	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD
Frogs and toads												
Acris blanchardi (NE/50)	0%	—	0%	—	0%	—	0%	—	86%	1.9 ± 2.7	0%	—
A. blanchardi (KS/50)	0%	—	0%	—	0%	—	0%	—	90%	0.9 ± 2.6	0%	—
Acris gryllus (MS/33)	0%	—	0%	—	0%	—	0%	—	63%	3.8 ± 3.6	0%	—
Bufo americanus (NE/3)	66%	3.6 ± 3.2	0%	—	0%	—	0%	—	0%	—	0%	—
B. americanus (MO/6)	50%	4.2 ± 5.3	33%	3.3 ± 1.1	0%	—	0%	—	0%	—	0%	—
Bufo fowleri (MS/8)	75%	4.6 ± 5.7	0%	—	0%	—	0%	—	25%	1.2 ± 2.1	0%	—
Bufo quercicus (MS/15)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
Bufo terrestris (MS/14)	100%	12.1 ± 10.6	0%	—	0%	—	0%	—	21%	1.7 ± 3	0%	—
Bufo woodhousii (NE/30)	0%		0%	—	0%	—	0%	—	27%	0.9 ± 2.7	0%	—
B. woodhousii (KS/30)	53%	4.6 ± 7.9	0%	—	0%	—	0%	—	50%	1.6 ± 1.1	0%	—
Gastrophryne carolinensis (MS /12)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
Hyla cineria (MS/18)	0%	—	0%	—	0%	—	0%	—	55%	2.1 ± 2.9	0%	—
Hyla chrysoscelis (NE/20)	0%	—	0%	—	0%	—	0%	—	20%	7.3 ± 10.1	0%	—
H. chrysoscelis (KS/20)	0%	—	0%	—	0%	—	0%	—	45%	2.6 ± 2.5	0%	—
Pseudacris triseriata (NE/87)	0%	—	0%	—	0%	—	0%	—	12%	0.3 ± 1.3	0%	—
P. triseriata (MO/5)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
Rana blairi (NE/21)	0%	—	0%	—	0%	—	0%	—	71%	6.7 ± 3.8	19%	0.9 ± 1.2
Rana catesbiana (NE/26)	0%	—	0%	—	0%	—	0%	—	7%	0.2 ± 0.2	0%	—
Rana clamitans (MS/11)	0%	—	0%	—	0%	—	0%	—	55%	1.7 ± 2.1	63%	2.6 ± 3.2
Rana sphenocephala (MO/6)	0%	—	0%	—	0%	—	0%	—	0%	—	100%	4.7 ± 3.1
R. sphenocephala (MS/33)	0%	—	0%	—	0%	—	0%	—	87%	4.5 ± 6.6	30%	1.1 ± 1.4
Rana sylvatica (MO/3)	0%	—	100%	7 ± 3.6	0%	—	0%	—	0%	—	0%	—
Spea bombifrons (NE/12)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
Snakes												
Agkistrodon piscivorous (MS/16)	0%	—	0%	—	25%	3.8 ± 1.2	0%	—	0%	—	0%	—
Coluber constrictor (NE/12)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
C. constrictor (MS/22)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
Lampropeltis getula (MS/8)	0%	—	0%	—	100%	5.4 ± 4.4	0%	—	0%	—	0%	—
Nerodia fasciata (MS/11)	0%	—	0%	—	0%	—	100%	4.5 ± 4	0%	—	0%	—
Pantherophis alleghaniensis (KS/5)	0%	—	0%	—	0%	—	40%	2.2 ± 3	0%	—	0%	—
Thamnophis sauritus (MS/6)	0%	—	0%	—	0%	—	100%	6.2 ± 6.2	0%	—	0%	—
Thamnophis sirtalis (NE/15)	0%	—	0%	—	0%	—	73%	3.5 ± 5.1	0%	—	0%	—
Thamnophis radix (NE/10)	0%	—	0%	—	0%	—	100%	5.2 ± 4.8	0%	—	0%	—

(Janovy Laboratory, University of Nebraska–Lincoln). Aged tap water was provided ad libitum. Hosts were given an appropriate temperature gradient via an under-the-tank heating pad and provided full spectrum lighting on a 12-hr day/light cycle.

Experimental infections of reptile and amphibian lungworms were conducted from July 2006 to October 2008. When groups of uninfected hosts became available they were exposed to sympatric lungworms. Trials of each lungworm species were conducted in succession to ensure host species were exposed to similar lungworms, i.e., worms collected from the same host species and location, to minimize issues of seasonality and local adaption. Every effort was made to standardize host-parasite exposures; time-0 and time-T controls were used in all trials to ensure hosts did not develop spurious infections through the course of the experiments. Time-0 controls were killed and necropsied at the beginning of the experimental infections, and time-T controls were maintained throughout the duration of the experiment, then killed and necropsied along with the experimental group. The experimental groups were maintained until infections should have developed according to Langford and Janovy (2009). At 30 days for *R. joaquinensis* and 40 days for the remaining 5 lungworm species, experimental groups were dissected, and all organs, muscles, body cavities, and connective tissues were thoroughly searched for lungworms. Prior to dissection, hosts were measured for SVL, and host sex was determined during dissection.

Prevalence and/or mean abundance were calculated as measures of parasitism for amphibians and reptiles examined (Margolis et al., 1982). Values are reported as mean ± 1 SD. Host specificity was calculated using 2 measurements: host range (HR), i.e., the number of host species a parasite can infect, and S_{TD}*, an unified index of host specificity that was developed by Poulin and Mouillot (2005) to combine phylogenetic and ecological data to quantitatively assess host specificity. S_{TD}* measures the average taxonomic distinctness among the host species used by a parasite, weighted for the parasite's prevalence in these different hosts (Poulin and Mouillot, 2005). Taxonomic distinctness represents the required number of steps along the hosts' taxonomic hierarchy to encounter a taxon that is common to 2 host species. For example, if a parasite infects two species in different genera but the same family, then 2 steps are required. Next, taxonomic distinctness values were weighted by the parasite's prevalence in each host species in a pair. If a parasite achieves high prevalence in a host, then more weight is given to the taxonomic distance between 2 host species. Finally, the index STD* is the ratio of the sum of the weighted taxonomic distinctness values to the sum of the weighting factors; the index is highest when a parasite obtains high prevalence values in distantly

related host species (see examples in Poulin and Mouillot, 2005). In this study the hosts' taxonomic hierarchy follows the recommendations of Pauly et al. (2009). $S_{TD}*$ has proven a useful calculation in comparative studies (e.g., Edwards and Vedrine, 2006), especially when parasite species are not collected from the same location and/or exposed to different sets of host species; our goal was to determine the usefulness of these indices for lungworms.

Extraction, PCR, and sequence analysis

Genomic DNA was extracted according to Tkach and Pawlowski (1999) using DNeasy tissue kits (Qiagen, Valencia, California), from single adult worms collected from the lungs. DNA fragments of approximately 2200 base pairs and spanning the 3′ end of 18S nuclear rDNA gene, internal transcribed spacer (ITS) region (ITS1_5.8S_ITS 2), and 5′ end of the 28S (including variable domains D1–D3) were amplified by polymerase chain reaction (PCR) on an Eppendorf Master Gradient thermal cycler. PCR reactions were performed in a total volume of 51.5 ml (42 ml H_2O, 5 ml Taq buffer, 1 ml dNTP at concentration 10 pM, 1 ml of each primer at concentration 10 pM, 0.25 ml of Taq, and 1.0–1.5 ml of template DNA solution, depending on the DNA concentration) by using Eppendorf Taq polymerase (concentration 5 units/1 ml). The Rhabdiasidae-specific forward primer ritf (59-GCGGCTTAATTTGACTCAA-CACGG-39) and the universal reverse primer 1500R (59-GCTATCCTGAGGGAAACTTCG-39) were used for both amplification and sequencing. In addition, internal primers ITS5 (59-GGAAG-TAAAAGTCGTAACAAGG-39), ITS4 (59-TCCTCCGCTTA TTGA-TATGC-39), 300R (59-CAACTTTCCCTCACGGTACTTG-39), and ECD2 (59-CTTGGTCCGTGTTTCAAGACGGG-39) were used for sequencing. PCR products were purified directly using Qiagen QIAquick columns (Qiagen, Valenica, California). Sequences were determined directly from PCR templates by cycle sequencing using Big Dye fluorescent dye terminators and protocols and an ABI 377 automated sequencer (Perkin-Elmer, Foster City, California). Sequences for 6 lungworms species were assembled using Sequencher (version 4.1.4, GeneCodes Corporation, Ann Arbor, Michigan) and provisionally aligned using ClustalX with default settings (Thompson et al., 1997). The resultant sequence alignment was then edited by eye with Bio Edit Sequence Alignment Editor (Hall, 1999) to remove ambiguous regions that appeared to lack homology, and the ends of each fragment were cut to match the shortest sequence in the alignment, yielding an 2104 character alignment. Sequences were submitted to GenBank under the following accession numbers: JX826435, JX826437, JX826438, JX826439 (*R. americanus*); JX826433 (*R. bakeri*); JX826441 (*R. eustreptos*); JX826442 (*R. fuscovenosa*); JX826430, JX826431, JX826432, JX826434, JX826443 (*R. joaquinensis*); JX826436, JX826440, JX826444 (*R. ranae*). Additional sequences acquired from GenBank, DQ264771.1 (*R. ranae*) and DQ264767.1 (*R. bakeri*) were included in the alignment.

Phylogenetic analysis

Using PAUP_4.0b10 (Swofford, 1998) maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on the combined, ITS, and LSU datasets. MP was performed using a heuristic search with unweighted and unordered characters. All searches included 100 stepwise random addition replicates with tree bisection-reconnection branch (TBR) swapping and a maximum of 100 trees. A strict consensus tree was constructed from the MP analysis when more than 1 best tree was found. Using Modeltest v. 3.06 (Posada and Crandall, 1998) a general time reversible model (GTR+I+G; Rodriguez et al. 1990) of substitution was selected for use in ML analysis. All sequences were added to the tree using 100 random addition replicates with the TBR branch-swapping algorithm. To establish support for nodes, 1,000 bootstrap replicates were calculated using heuristic search criteria for both MP and ML analyses. Gaps were treated as missing data in both searches, and analyses were unrooted because no appropriate, alignable outgroup was available.

RESULTS

Amphibian and reptile field studies

Over a 2-yr period, more than 1,700 lungworms were recovered from the lungs of 615 amphibians and reptiles (Table I). *Rhabdias eustreptos* (HR = 2, $S_{TD}* = 3$) and *R. fuscovenosa* (HR = 5, $S_{TD}* = 1.6$) were found infecting only snakes. Snake lungworms did not infect all available host species in nature, only a subset of snake species collected at each location. The remaining 4 lungworm species infected only anurans, i.e., frogs and toads; however, host specificity in nature varied greatly among these lungworm species: *R. americanus* (HR = 4, $S_{TD}* = 1$) infected only species of Bufonidae, *R. bakeri* (HR = 2, $S_{TD}* = 3$) infected 1 species of Bufonidae and 1 species of Ranidae, *R. joaquinensis* (HR = 12, $S_{TD}* = 2.34$) infected 4 different host families, and *R. ranae* (HR = 4, $S_{TD}* = 1$) infected only species of Ranidae. Finally, 3 species of anurans were not infected at any location, i.e., *B. quercicus*, *G. carolinensis*, and *S. bombifrons*, nor was the snake *C. constrictor* infected by either species of snake lungworm.

Phylogenetic analysis

Nucleotide frequencies for the combined rDNA data set were 0.242 (A), 0.202 (C), 0.270 (G), and 0.283 (T); frequencies were not significantly heterogeneous across taxa ($X^2 = 32.55$, $P = 0.67$). Of 2,104 bp, 1m901 were invariant, 16 variable characters were parsimony uninformative, and 187 were parsimony informative (149 from the ITS dataset). Maximum parsimony analysis yielded 2 trees with a consistency index (C.I.) of 0.95 and a length of 226 steps. Both the MP and ML trees supported distinct clades for anuran and snake lungworms (Fig. 1). The methods also agreed upon species designations, including 1 possible cryptic species, *R. ranae* collected in Mississippi. In the ML analysis, *R. ranae* from Mississippi were placed as a sister species to *R. joaquinensis*, instead of grouping with *R. ranae* collected from Nebraska and North Dakota. To avoid potential complications due to cryptic species, host specificity experiments in this study regard *R. ranae* from Mississippi as a distinct population.

The LSU dataset (935 bp) provided poor phylogenetic signal compared to the ITS dataset. Forty sites in the LSU dataset were variable among all lungworm species; however, only 11 variable sites occurred among lungworm species that infect anurans. A strict consensus of 14 MP trees and the ML tree yielded polytomys for anuran lungworms (data not shown). However, despite the poor phylogenetic signal, both MP and ML analyses supported distinct clades for anuran and snake lungworms.

The ITS dataset provided a satisfactory phylogenetic signal. For ITS-1 (249 bp), 84 sites were variable, but only 29 sites varied among lungworms collected from anurans. The 200 bp ITS-2 dataset contained 70 variable sites among all lungworms, but only 17 among worms that infect anurans. Maximum parsimony analysis of the ITS dataset yielded 1 tree with a C.I. of 0.96 and a length of 184 steps (Fig. 2). The topologies of the ML and MP analyses from the ITS dataset agreed. The tree supports a sister species relationship between *R. ranae* from Mississippi and *R. joaquinensis* from Mississippi, Nebraska, and Kansas during the combined analysis but disagrees with the combined analysis by placing *R. ranae* as a recent common ancestor to *R. ranae* from Mississippi and *R. joaquinensis*. The ITS analysis places *R. bakeri* basal to all other anuran lungworms, which disagrees with the sister species relationship observed in the combined analysis. The ITS dataset analysis agrees with both the combined and LSU datasets that anuran and snake lungworms form distinct clades.

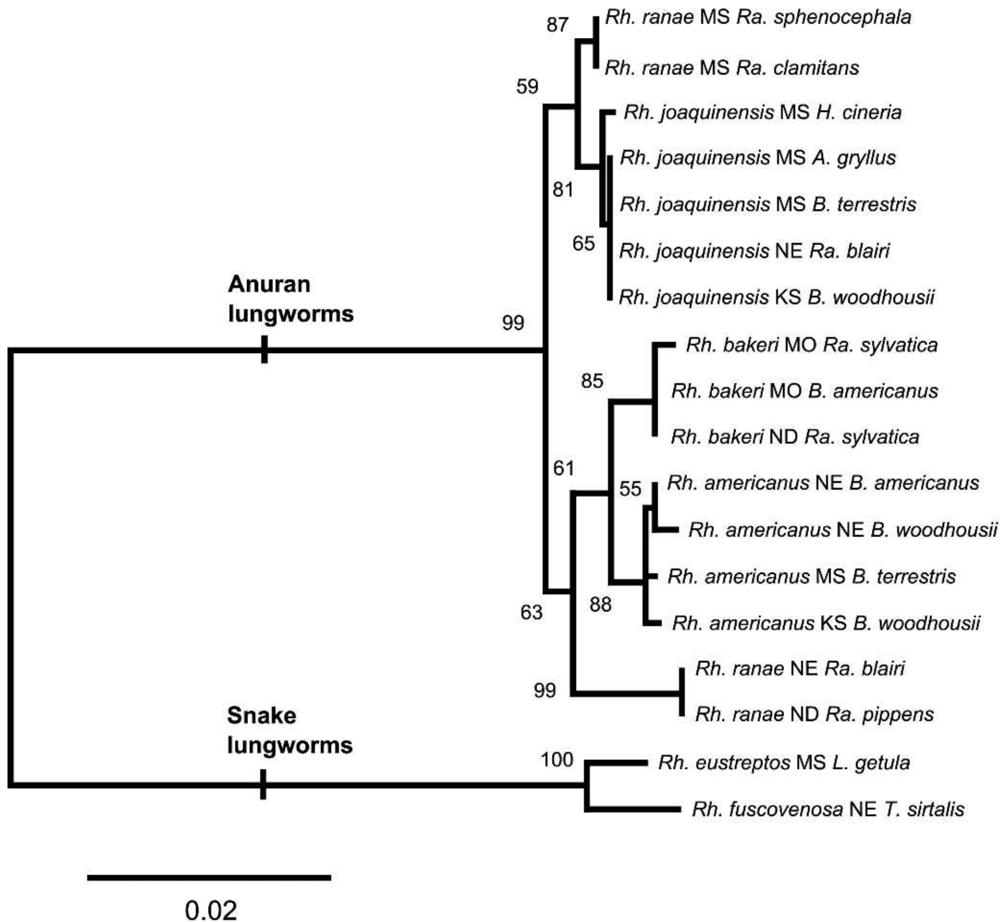

FIGURE 1. Lungworm maximum likelihood tree (unrooted) based on entire rDNA dataset (partial LSU, ITS1, ITS2). Branch lengths equal substitutions/site. Bootstrap values are given as percentages near the individual nodes, with 1,000 bootstrap replicates using heuristic search criteria. Lungworm branches end in lungworm species, collection location, and host species names to provide information about host specificity. Kansas (KS), Mississippi (MS), Missouri (MO), Nebraska (NE), and North Dakota (ND).

Experimental infections

Infective juveniles of the 4 anuran lungworms species were unable to establish infections in salamanders or snakes and varied in their ability to infect anurans (Table II). *Rhabdias americanus* (HR = 3, $S_{TD}* = 1.78$) infected all *Bufo* spp. and exhibited a high prevalence of 100% and mean abundance of 28.1 ± 1.6 in *B. terrestris*. These lungworms also infected *Rana sylvatica* with a prevalence of 57% and mean abundance of 12.0 ± 12.7. *Rhabdias bakeri* (HR = 2, $S_{TD}* = 3$) infected *Rana sylvatica* with a prevalence of 100% and mean abundance of 12.0 ± 11.7 but was unable to establish infections in *Rana sphenocephala*. However, the toad, *B. americanus*, was infected by *R. bakeri* with a prevalence of 71% and mean abundance of 11.2 ± 9.3. *Rhabdias joaquinensis* (HR = 8, $S_{TD}* = 2.7$) infected all anurans to which it was exposed, except *G. carolinensis*, which was resistant to all lungworm exposures. Prevalence reached a high of 100% in 3 host species, and mean abundance reached a high of 28.1 ± 2.4 in *Rana blairi*. *Rhabdias ranae* from Nebraska (HR = 2, $S_{TD}* = 1$) infected only leopard frogs, *Rana blairi* and *Rana pipiens*, reaching 100% prevalence in both species and a maximum mean abundance of 28.5 ± 4.0 in the former host species. *Rhabdias ranae* from Mississippi (HR = 4, $S_{TD}* = 2.3$) displayed reduced host specificity when compared to worms from Nebraska. The worms infected 2 *Rana* spp. and 2 *Bufo* spp., with a high prevalence of 100% and mean abundance of 24.1 ± 5.4 in *Rana sphenocephala* but were unable to infect species of *Acris* or *Hyla*.

Experimental exposures of snake lungworms confirmed that *R. eustreptos* and *R. fuscovenosa* were unable to infect amphibians or turtles (Table III). However, *R. fuscovenosa* was able to establish an infection in a lizard, with 1 of 5 *S. undulatus* infected with 15 gravid worms. Both snake lungworm species infected all snakes to which they were exposed, with a prevalence of 100% in several host species; mean abundance reached a high of 28 ± 2 for *R. eustreptos* in *L. getula* and 26.6 ± 4.2 for *R. fuscovenosa* in *C. constrictor*. None of the time-0 or time-T controls was infected with any lungworm species (anuran or snake) for any experiment.

DISCUSSION

Our study combines field studies and experimental infections with a parasite phylogeny to comparatively establish host specificity in North American lungworms. Prior to this study, snake lungworm host specificity had not been formally addressed (but see Chu, 1936), and anuran lungworms were assumed to be strictly host specific based on field studies (Kuzmin et al., 2003, and references within). These assumptions likely over-estimated host specificity, i.e., assumed a decreased host range, due to the intractable problems associated with determining host specificity with field studies alone. Our study challenges universal strict host specificity in anuran lungworms and establishes lungworms collected from snakes as relative generalists. Furthermore, our research establishes 2 distinct clades in North Ameican lungworms: (1) *R. eustreptos* and *R. fuscovenosa* as parasites of snakes

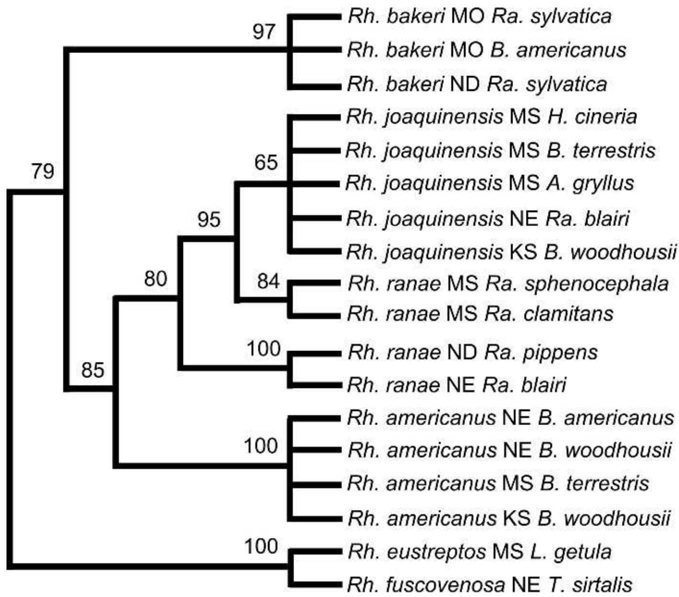

FIGURE 2. Maximum parsimony tree (unrooted) based on ITS dataset, maximum likelihood analysis agreed on tree topology. Bootstrap values are given as percentages near the individual nodes, with 1,000 bootstrap replicates using heuristic search criteria. Lungworm branches end in lungworm species, collection location, and host species names to provide information about host specificity. Kansas (KS), Mississippi (MS), Missouri (MO), Nebraska (NE), and North Dakota (ND).

and (2) *R. americanus*, *R. bakeri*, *R. joaquinensis*, and *R. ranae* as parasites of anurans. In addition, this study is the first to suggest ecological fitting plays a role in shaping lungworm host specificity. We also found little evidence to support progressive specialization, sensu Brooks and McLennan (2003), in the genus *Rhabdias*. Taken together, the results of our combined approach suggest that ecological interactions and in some instances physiological constraints are the primary drivers of host specificity in natural populations of North American *Rhabdias* spp.

The ITS region of rDNA has been shown to be a useful molecular region for investigating evolutionary patterns among nematodes (Powers et al., 1997). Here, we found that the ITS region was variable and thus provides a good phylogenetic signal, whereas the LSU dataset was shown to have a relatively poor phylogenetic signal. This suggests that our combined analysis may suffer from an over-abundance of invariable sites. Despite variations in phylogenetic signals, all 3 datasets revealed separate clades for anuran and snake lungworms. The genetic distance between snake and anuran lungworms suggests these 2 clades may need to be reclassified into separate genera. However, data from other rhabdiasid species are needed before this assertion can be confirmed. Separation of these 2 clades is supported by their different host specificities (this study) and life cycles (Langford and Janovy, 2009). The phylogeny also supports our species identifications and alleviates concerns about cryptic species, except among *R. ranae*.

The combined dataset supports *R. americanus* and *R. bakeri* as sibling taxa. However, the ITS dataset also places *R. bakeri* basal to all other lungworm species that infect anurans. This close relationship between the 2 worm species may partially explain the ability of *R. bakeri* to infect toads; moreover, the relationship suggests a host switch from frogs (Ranidae) to toads (Bufonidae) (or vice versa). The ITS and combined datasets agree on the distinct phylogenetic signal of *R. ranae* collected from Nebraska/North Dakota (herein referred to as *R. ranae* from the Midwest) versus Mississippi (herein referred to as *R. ranae* from the Southeast). The ITS and combined datasets agree that *R. ranae* from the Southeast and *R. joaquinensis* are sibling taxa, and, according to the ITS dataset, the 2 species share a common ancestor with *R. ranae* from the Midwest. However, the combined dataset places *R. ranae* from the Midwest in a clade with *R. bakeri* and *R. americanus*. Given the morphological similarity between *R. ranae* from the Midwest and *R. ranae* from the Southeast, it seems more likely that the relationship dictated by the ITS dataset will be confirmed with additional sequence data.

The host specificity of lungworms is a controversial issue. A recent study by Tkach et al. (2006) concluded that *R. bakeri* displayed strict host specificity, infecting only *Rana sylvatica*; however, earlier experimental infections conducted by Baker (1979) showed *R. bakeri* was able to infect *B. americanus*. Likewise, a study by Kuzmin et al. (2003) considered *R. americanus* to be a parasite strictly of toads (*Bufo* spp.), despite Baker's (1979) previously successful cross-transmission studies that showed *R. americanus* could infect *Rana sylvatica*. In both cases, our results corroborate Baker's findings. Our experimental infections established *R. americanus* infections in both *Bufo* spp. and *Rana sylvatica*, but only *Bufo* spp. were found infected in nature. Based on successful experimental infections, we consider *Rana sylvatica* a viable host for *R. americanus* and suggest that future field studies will find the lungworm infecting wild *Rana sylvatica* in habitats conducive to sympatry of *Rana sylvatica* and *Bufo* spp. Similarly, field studies in Missouri found *R. bakeri* infecting *Rana sylvatica* and the distantly related toad *B. americanus*. These results were replicated in our experimental infections; therefore, we consider *B. americanus* a viable host for *R. bakeri*. Interestingly, we were unable to infect *Rana sphenocephala*, a close relative of *Rana sylvatica* (both frogs are members of the subgenus *Aquarana*). We found infective juvenile *R. bakeri* were able to penetrate *Rana sphenocephala*, but worms never progressed further than the body cavity; whether the host immune system prevented development or worms became lost during migration is unclear. Host macrophages were present on worms, but this observation is not uncommon for migrating worms in normal infections. The ability of *R. bakeri* to infect a distantly related host, i.e., separate families, but not *Rana sphenocephala*, suggests that these worms are tracking host resources instead of host phylogenies, i.e., they are an example of ecological fitting (sensu Brooks et al., 2006). Similarly, the ability of multiple *Rhabdias* spp., which are dispersed throughout our phylogeny, to infect multiple species of unrelated hosts provides evidence that progressive specialization is not common in the genus *Rhabdias* (see Brooks and McLennan, 2003).

Our suggestion that *R. bakeri* is tracking host resources is supported by the unique terrestrial behavior of *Rana sylvatica*, which brings it into close contact with toads, but not other frogs. The majority of *Rana* spp. are aquatic or semi-aquatic, rarely spending extended time periods in terrestrial environments, but *Rana sylvatica* has a distinctly terrestrial life style, inhabiting landscapes similar to most *Bufo* spp. (Conant and Collins, 1998). Inhabiting terrestrial habitats likely exposes *B. americanus* and *Rana sylvatica* to each other's feces, thus parasite transmission occurs, whereas *Rana sylvatica* only shares habitat with other

TABLE II. Prevalence (P) and mean abundance (MA) ± standard deviation (SD) from 4 *Rhabdias* spp. experimentally exposed to sympatric amphibians and reptiles. Hosts were exposed to 30 infective lungworms for 12 hr. NE = Nebraska, MO = Missouri, MS = Mississippi. *Rhabdias ranae* is split into 2 populations, Mississippi and Nebraska.

Experimental species (location)	*R. americanus* MS		*R. bakeri* MO		*R. joaquinensis* NE		*R. ranae* NE		*R. ranae* MS	
	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD
Frogs and toads										
Acris blanchardi (NE)					100% (34)	26.7 ± 7.5				
Acris gryllus (MS)	0% (7)	—							0% (5)	—
Bufo americanus (NE)							0% (18)	—		
B. americanus (MO)			71% (14)	11.2 ± 9.3						
Bufo fowleri (MS)	75% (12)	18.4 ± 7.1							50% (15)	3.9 ± 13.2
Bufo quercicus (MS)	94% (34)	24.5 ± 7.8								
Bufo terrestris (MS)	100% (33)	28.1 ± 1.6							64% (14)	6.9 ± 6.8
Bufo woodhousii (NE)					95% (58)	25.9 ± 5.2	0% (18)	—		
Gastrophryne carolinensis (MS)	0% (5)	—			0% (7) †	—			0% (4)	—
Hyla cineria (MS)	0% (7)	—							0% (5)	—
Hyla chrysoscelis (NE)					93% (30)	20.3 ± 10.2				
Pseudacris triseriata (NE)					45% (22)	8.1 ± 12.7	0% (20)	—		
P. triseriata (MO)			0% (10)	—						
Rana blairi (NE)					100% (26)	28.1 ± 2.4	100% (16)	28.5 ± 4.0		
Rana catesbiana (MS)	0% (10)	—							0% (11)	—
R. catesbiana (NE)					10% (30)	2.7 ± 8.2	0% (20)	—		
Rana clamitans (MS)	0% (12)	—							75% (4)	11 ± 10
Rana pipiens (NE)					100% (8)	24.6 ± 6.6	100% (21)	26.4 ± 3.5		
Rana sphenocephala (MS)	0% (12)	—							100% (35)	24.1 ± 5.4
R. sphenocephala (MO)			0% (11)	—						
Rana sylvatica (MO)	57% (7)*	12 ± 11.7	100% (32)	26.4 ± 4.2						
Spea bombifrons (NE)					83% (6)	17.3 ± 9.8	0% (5)	—		
Salamanders										
Ambystoma tigrinum (NE)					0% (7)	—	0% (5)	—		
A. tigrinum (MS)	0% (4)	—							0% (3)	—
Snakes										
Agkistrodon piscivorous (MS)	0% (2)	—							0% (2)	—
Pantherophis alleghaniensis (NE)					0% (4)	—	0% (2)	—		
Thamnophis sauritus (MS)	0% (6)	—							0% (3)	—
Thamnophis sirtalis (NE)					0% (6)	—	0% (3)	—		

* *Rhabdias americanus* collected from MO *Bufo americanus*.
† *Rhabdias joaquinensis* collected from MS *Acris gryllus*.

TABLE III. Prevalence (P) and mean abundance (MA) ± standard deviation (SD) from 2 snake *Rhabdias* spp. experimentally exposed to sympatric amphibians and reptiles. Hosts were given 30 infective lungworms. NE = Nebraska, MS = Mississippi.

	R. eustreptos MS		*R. fuscovenosa* NE	
Experimental species (location)	P (n)	MA ± SD	P (n)	MA ± SD
Snakes				
Agkistrodon piscivorous (MS)	100% (5)	21.2 ± 5.2	100% (3)*	18 ± 5.3
Coluber constrictor (NE)			100% (5)	26.6 ± 4.2
C. constrictor (MS)	100% (6)	20 ± 3.4		
Pantherophis alleghaniensis (NE)			100% (4)	22.5 ± 3.5
Lampropeltis getula (MS)	100% (4)	28 ± 2		
Nerodia fasciata (MS)	75% (8)	13.9 ± 10.7		
Thamnophis sauritus (MS)	50% (8)	8 ± 7.1		
Thamnophis sirtalis (NE)			100% (8)	20 ± 5.7
Lizards				
Aspidoscelis sexlineatus (NE)			0% (5)	—
Sceloporus undulatus (NE)			20% (5)	15
S. undulatus (MS)	0% (4)	—		
Turtles				
Kinosternon subrubrum (MS)	0% (3)	—		
Trachemys scripta (MS)	0% (4)	—		
Frogs and toads				
Bufo woodhousii (NE)			0% (10)	—
Rana blairi (NE)			0% (7)	—
Rana catesbiana (MS)	0% (15)	—		
Rana sphenocephala (MS)	0% (6)	—		
Spea bombifrons (NE)			0% (3)	—

* *Rhabdias fuscovenosa* collected from Mississippi *Nerodia fasciata*.

Rana spp. during the breeding season. Furthermore, *Rana sylvatica* possesses physiological aspects, such as epidermal properties, e.g., desiccation times (Schmid, 1965) that are toad-like, which may facilitate parasite transmission between the 2 species. In other words, the lungworm may not differentiate between the 2 species because they offer somewhat interchangeable habitats, but the hosts must inhabit the same geographic space for cross-infection to occur.

Although the habitats provided by host species appear to be somewhat interchangeable, i.e., amenable to lungworm establishment, they are likely not identical. If each host provides an identical habitat, then we would expect equivalent infection parameters; however, we found greater prevalence and higher mean abundance in *R. bakeri* infecting *Rana sylvatica* when compared to *B. americanus*. These results suggest that a majority of *R. bakeri* in nature cycle through *Rana sylvatica*, but the importance of infections in *B. americanus* should not be discounted. Infections in toads may represent an ongoing or past host switch and could provide the nematode with future evolutionary avenues. For example, in the event that *Rana sylvatica* are extirpated in Missouri, *R. bakeri* may be able to subsist and adapt to exclusively infecting *Bufo* spp. Furthermore, spatiotemporal variations in host species' relative abundances may result in a majority of lungworms cycling through *B. americanus* instead of *Rana sylvatica* during different seasons and/or years. Such switches may be oscillatory, where parasite host range episodically alternates between hosts through time (see Janz and Nylin, 2007; Hoberg and Brooks, 2008). Finally, based on infections in both host species, we know that *Rana sylvatica* and *B. americanus* in Missouri share geographic space, but it is not clear if the habitat of these hosts sufficiently overlap to allow for cross-transmission in nature in other portions of their range. Further exploration of host habitat usage and additional field collections may shed light on these differences.

The lungworm *R. joaquinensis* has been previously collected from several *Rana* spp. and *B. americanus* (see Kuzmin et al., 2003). Our field studies and experimental infections found *R. joaquinensis* capable of infecting numerous species of anurans in several families. We consider the lungworm to be a true generalist, infecting virtually all North American anurans it encounters, although some anurans, e.g. *Spea* spp., probably rarely encounter lungworms due to their unique life histories. The only anuran *R. joaquinensis* could not infect was the microhylid, *G. carolinensis*, which was resistant to all lungworm experimental infections in this study, and in which natural lungworm infections have never been found. Upon exposure to infective juvenile lungworms, *G. carolinensis* secrete a mucus substance over their entire body that evidently impedes lungworm skin penetration (G. J. Langford, pers. obs.). We also suspect that the poison glands of *G. carolinensis* are activated by attempted lungworm penetration, which has previously been suggested as an anti-predatory mechanism (Garton and Mushinsky, 1979).

The bullfrog, *Rana catesbiana*, also appears to be a physiologically resistant host. While we did establish infections with low mean abundance with *R. joaquinensis* in young male bullfrogs from Nebraska, these worms contained relatively few gravid eggs

in comparison to worms from control infections in *Rana blairi* and *Acris crepitans*. Thus, our experimental infections and field surveys suggest that young bullfrogs, but not healthy adults, may become infected with gravid lungworms in Nebraska. Furthermore, bullfrogs may represent evolutionary dead ends because they primarily defecate in water (G. J. Langford, pers. obs.), thus drowning all lungworm offspring, which are incapable of swimming.

Morphologically similar *R. ranae* from Mississippi and Nebraska displayed different host specificity tendencies in nature and in the laboratory. *Rhabdias ranae* from the Midwest displayed the strictest host specificity for all lungworm species in this study, which supports the Tkach et al. (2006) findings that wild *R. ranae* from the Midwest (North Dakota) are strictly parasites of leopard frogs. Thus, it appears that *R. ranae* from the Midwest are leopard frog specialists. Conversely, we suggest *R. ranae* from the Southeast are tracking a broader set of host resources and thus can infect non-leopard frog *Rana* spp. and potentially toads in nature. However, as mentioned above, toads and most *Rana* spp. (*Rana sylvatica* is an exception), including the semi-aquatic *Rana sphenocephala* and *Rana clamitans*, do not share habitat for extended periods of time (sharing may occur during the breeding season), minimizing the likelihood of cross-transmission of *R. ranae* from the Southeast between toads and frogs.

Experimental infections with snake lungworms, *R. eustreptos* and *R. fuscovenosa*, indicate that they are each capable of infecting all North American snake species, whereas in nature our studies show that *R. eustreptos* infected only *L. getula* (Colubridae) and *A. piscivorous* (Viperidae), and *R. eustreptos* is a generalist infecting several species of Colubridae (Table III). The absence of infections in wild hosts is likely ecologically mediated; unfortunately, because snake lungworms infect hosts orally, potentially via transport hosts, e.g., snails or frogs (Langford and Janovy, 2009), deducing likely ecological constraints on lungworm infections is difficult. Despite these limitations, we noticed that neither *R. eustreptos* nor *R. fuscovenosa* infected field-collected *C. constrictor*, yet laboratory exposures always established infections. The terrestrial lifestyle of *C. constrictor* may preclude infection. Further, strictly terrestrial snakes are less likely to consume potential transport hosts. For example, *C. constrictor* stomach contents in this study were primarily composed of terrestrial arthropods, e.g., orthopterans, which are not suitable transport hosts (see Langford and Janovy, 2009). The remaining snake species in this study frequent aquatic sites (Ernst and Ernst, 2003) and consume potential transport hosts, which may explain why aquatic and semi-aquatic snakes are infected in nature.

Unexpectedly, we found *R. fuscovenosa* was able to infect the lizard *S. undulatus* under laboratory conditions. Prior to this study, snake lungworms had not been recovered from lizards, although a few species of *Rhabdias* have been discovered infecting lizards outside of the United States and Canada (Bursey et al., 2003, 2007). Upon dissection, it was noted that the lizard was cold to the touch, and the tank-heating system was not functioning. It is possible that the infection was a consequence of suboptimal temperatures preventing the basking behavior known to combat infections in other reptiles (Kluger, 1979; Carey et al. 1999).

Overall, measurements of host specificity determined in this study, HR and S_{TD}*, agree with our conclusion that host specificity varies greatly among species of *Rhabdias*. As we suspected, snake lungworms displayed little host specificity, whereas anuran lungworms ranged from strict host specificity, e.g., *R. ranae* from Nebraska, to relative generalist, e.g., *R. joaquinensis*. While these indicies of host specificity are not perfect, e.g., we do not consider *R. bakeri* ($S_{TD}^* = 3.0$) to have less host specificity than *R. joaquinensis* ($S_{TD}^* = 2.7$), they do provide a generally accurate depiction of lungworm host specificity, and incongruences between HR and S_{TD}* point to interesting host-parasite relationships that need further study. For example, the snake lungworm *R. eustreptos* (HR = 2, $S_{TD}^* = 3$) has a relatively low HR and high S_{TD}* in comparison to *R. fuscovenosa* (HR = 5, $S_{TD}^* = 1.6$). In the laboratory, both lungworm species infected all snakes they were exposed to; thus it is confusing that *R. eustreptos* infects only 2 host species in nature in Mississippi. Similarly, it is peculiar that *R. fuscovenosa* infects several host species in nature, but only within the Colubridae and mostly natricines. In other words, what ecological interactions are preventing lungworms from realizing their fundamental host range in nature? Such questions concerning lungworm host specificity could form the basis for challenging ecological research in the future.

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