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RECOGNIZING THE CAUSES OF PARASITE MORPHOLOGICAL VARIATION TO RESOLVE THE STATUS OF A CRYPTOGENIC PENTASTOME

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ABSTRACT

Exotic species can threaten biodiversity by introducing parasites to native hosts. Thus, it is critical to identify if the same parasite species infects both native and exotic hosts. However, developmental- or environmental-induced morphological variation may render species identification ambiguous. Our study reports a range expansion in the southern United States of the pentastome *Raillietiella indica* from the Mediterranean gecko, *Hemidactylus turcicus*, as well as a host expansion into the green anole, *Anolis carolinensis*, in the anole's native range. Species identification was based on sequence data and male spicule shape. In agreement with a study from Australia, we found that much of the morphological variation in hook measurements, the primary diagnostic traits of raillietiellid pentastomes, was due to development. Here, we explicitly link this developmental variation to instar stage by incorporating experimental infection data obtained from the literature. We also show that the various hook traits are themselves highly correlated and, thus, likely not independent. Taking instar stage and correlated hook variables into account, we directly controlled for development on a composite hook size measurement. Using a large sample size from *H. turcicus*, we did not find any consistent effects of potential factors (host sex, host snout-vent-length, or parasite intensity) that may result in environmental-induced variation in relative hook size (corrected for body length). However, relative male spicule size tended to be negatively correlated with parasite intensity. In contrast, both pentastome body length and relative hook size significantly varied among host species whereas relative male spicule size was not significantly different among host species. Our study independently supports the conclusions that developmental- and host-induced morphological variations need to be accounted for to accurately identify pentastome species.

Approximately 42% of native species listed as endangered or threatened in the United States are at risk due to invasive species (Pimentel, 2011). Free-living invasive species are often studied for their direct ecosystem impacts on nutrient cycling or habitat structure (Simberloff, 2011). However, there could also be indirect impacts (e.g., apparent competition via a shared parasite) for which the effects may be more subtle, but not necessarily inconsequential, in driving changes in the native community (Simberloff, 2011). One possible indirect effect can arise from spillover of co-invasive parasites—where an exotic host brings an exotic parasite and the parasite subsequently infects native hosts (Kelly et al., 2009; Lymbery et al., 2014). The conservation concern in spillover is that an invasive parasite represents a novel infection that could reduce the fitness of native hosts and, hence, negatively impact the native community (Dobson and May, 1986; Daszak et al., 2000). Another possibility is spillback, where an exotic host acquires a native parasite and subsequently amplifies the parasite population in native hosts (Kelly et al., 2009).

A critical initial step in studying the potential impacts of parasites in species invasions is to determine if there is a shared parasite between native and alien hosts and, if so, determine if the parasite is native or alien. Unfortunately, determining whether a parasite is native or introduced can be problematic, and in such cases parasites are given cryptogenic status, i.e., alien or native status cannot be ascertained (Carlton, 1996). For example, it can be difficult to resolve the origin of parasites if they were anthropogenically transported prior to taxonomic surveys (Carlton, 1996; Criscione and Font, 2001a). Many parasite species are also designated as cryptogenic because of taxonomic ambiguity (Lymbery et al., 2014). Parasites may not be identified to species or misidentified because of a limited number of measurable morphological traits, investigator-induced phenotypic variation during specimen handling/preparation, extensive underlying

genetic variation of phenotypes, or environmental-induced (especially host-induced) phenotypic variation (Riley, 1986; Criscione and Font, 2001b; Perkins et al., 2011). Clarifying cryptogenic species as alien or native is important for understanding several aspects of biological invasions such as identifying invasion corridors, susceptibilities of communities to invasions, and frequencies of introductions and successful invasions (Carlton, 1996). Moreover, establishing if a parasite is native or alien allows differentiation between spillover and spillback effects.

The subject of our study is a pentastome parasite that infects the lungs of the invasive Mediterranean gecko, *Hemidactylus turcicus*, in the southern United States. Prior to our study, there were reports of 2 species of pentastomes infecting *H. turcicus* in the continental United States: *Raillietiella frenatus* [sic] in Hidalgo, Texas (Pence and Selcer, 1988) and *Raillietiella teagueselfi* newly described in Houston, Texas (Riley et al., 1988). However, Kelehear et al. (2011) recently demonstrated ambiguities in interpreting key taxonomic traits of raillietiellid pentastomes, calling into question the species identifications of past studies. Their study found that anterior and posterior hook measurements were correlated with pentastome body size, which indicated that hook size covaried with development. Using DNA sequence data, Kelehear et al. (2011) concluded that the same pentastome species infected 2 exotic host species, *Hemidactylus frenatus* (Asian house gecko) and *Rhinella marina* (cane toad), and the native tree frog *Litoria caerulea* in Australia (Kelehear et al., 2011). They identified the raillietiellid species infecting these 3 hosts as *R. frenatus* [sic] (correct spelling should be *R. frenata* as discussed in Poore, 2012). However, Poore (2012) lists *R. frenata* as a junior synonym to *Raillietiella indica*. Therefore, we refer to the pentastome as *R. indica* henceforth.

Given the previous reports in the United States, we hypothesized that the pentastome infecting *H. turcicus* in the southern United States was *R. indica*. We followed the advice of Kelehear et al. (2011) that species identifications should account for morphological variation due to parasite developmental stage. However, our approach differs from the latter study in 2 key aspects of the analysis. First, we tested if hook measurements were themselves highly correlated to determine whether they may represent a single trait. Second, using traditional raillietiellid morphometric analyses along with prior life cycle work (Ali and Riley, 1983), we a priori assigned individual pentastomes to distinct instar stages. Taking instar stage and correlated hook variables into account, we directly tested the role of, and subsequently controlled for, development on a composite hook measurement. In addition, a large sample size of pentastomes from *H. turcicus* enabled us to test if additional factors such as host body size, host sex, and parasite density-dependence influenced hook morphology. Lastly, by combining data from our study and Kelehear et al. (2011), we tested for broader host species effects on pentastome morphology. Based on the morphological results and DNA sequence data, we report a range expansion of the alien *R. indica* from the Mediterranean gecko in the southern United States as well as a host expansion into the green anole, *Anolis carolinensis*, in the anole's native range.

MATERIALS AND METHODS

Sampling

Geckos were captured by hand from locations in Metairie, Louisiana (30°0.76′N, 90°8.90′W); Ingleside, Texas (27°52.05′N, 97°12.75′W); and Port Aransas, Texas (27°50.183′N, 97°3.117′W) from 2011–2013. Anoles were captured from a location in Metairie, Louisiana in 2012. Additional details on the sampling locations are given in Caballero et al. (2015) and Criscione and Font (2001c). Data recorded from lizard hosts included weight, total length, snout-vent-length (SVL), and sex. The research protocols, i.e., capture, handling, and sacrifice (decapitation followed by pithing) prior to dissection in this study were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Live pentastomes were recovered from the lungs, placed in 0.7% saline solution, and then placed at 4 C for a few minutes to relax them. Next, 90 C water was poured on the pentastomes to heat-kill and fix. Pentastomes were then stored in 70% ethanol at 4 C.

DNA extraction and amplification

We sequenced 24 pentastomes: 16 from *H. turcicus* (8 from Metairie, Louisiana; 2 from Port Aransas, Texas; and 6 from Ingleside, Texas) and 8 from *A. carolinensis* from Metairie, Louisiana. For DNA extractions, a 1-mm^3 piece of tissue from an individual parasite was placed into 200 µl of 5% chelex containing 0.2 mg/ml of proteinase K. Samples were incubated at 56 C for 2 hr then boiled at 100 C for 8 min. As in Kelehear et al. (2011), we amplified the cytochrome *c* oxidase subunit 1 (*CO1*) of the mitochondria with the primer pair LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′)/HCO2189 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). PCR amplification was performed with an initial denaturation of 95 C for 3 min followed by 36 cycles of 94 C for 45 sec, 55 C for 30 sec, and 72 C for 45 sec, followed by a final extension of 72 C for 7 min. PCR products were purified with the Ultra Clean PCR clean-up Kit (MO BIO Laboratories, Inc., Solana Beach, California) and then sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, Connecticut) for Sanger sequencing. Sequences were aligned with Unipro UGENE (Okonechnikov et al., 2012).

Two male and 2 female whole voucher pentastome specimens are deposited in the Smithsonian Institution (USNM 1522647, 1522658, 1522662, 1522664). Tissue samples from 3 infected *Hemidactylus turcicus* are deposited in the Texas A&M Biodiversity Research and Teaching Collections (TCWC 104374, 104375, 104376). Our *CO1* pentastome sequence was deposited in GenBank (MK208827).

Morphological measurements

To identify the pentastomes in the gecko and anole hosts, we measured morphological traits typically found in species descriptions (Riley, 1986). Several measurements were conducted on the 2 pairs of anterior and posterior hooks that surround the buccal cavity at the anterior end of the worm. In addition to hook measurements, we measured body length and the size of the male copulatory spicules. General shape of the male copulatory spicules is also considered diagnostic. Although the number of annuli has also been included in species descriptions of raillietiellid pentastomes, we did not include this trait because several researchers have noted the limited diagnostic value of annuli counts (Riley, 1986; Kelehear et al., 2011).

All measurements were based on digital images of whole specimens or morphological traits using a Leica DM1000

compound scope (Leica Microsystems, Wetzlar, Germany) or a Nikon SMZ645 dissecting scope (Nikon Corporation, Tokyo, Japan) and a Nikon 5700 camera (Nikon Inc., Melville, New York). The pictures were analyzed with the segmented-line and straight line tools in ImageJ software (Schneider et al., 2012) where images of a micrometer scale taken at corresponding magnifications were used to calibrate pixels/millimeter per each magnification setting. Full body length measurements were done by taking pictures of the worms under a dissection microscope set to the highest magnification that allowed the entire worm to be visible. To take clear images of the hooks, the anterior end of the pentastome was removed and soaked in a lactophenol solution for 10 min to clear the tissue before the sample was mounted on a slide with glycerol under a cover slip (total magnification ×100). Body length measurements were taken with the segmented-line tool, keeping the line in the middle of the worm. Anterior and posterior hook measurements followed that of Ali et al. (1981) (see their fig. 5 or fig. 7 in Kelehear et al., 2011). Briefly, hook length measurements were split into 2 straight-line measurements: blade length (AB) and shank length (BC). AB measurements were from the tip of the hook's barb (point A) to the small projection formed where the hollow back closes (point B). BC measurements were taken from point B to the bottom of the hook's flared base (point C). Kelehear et al. (2011) introduced the new measure of hook bluntness, which is measured by taking the area at the tip of the hook. The area is estimated by outlining the edges of the hook tip from point A up to 20 μm along the hook shaft. In males, copulatory spicule length measures were taken by placing line points in the middle of the spicule from the base to the tip of the hook and width was taken at the widest part of the base (total magnification ×100). Because the traits above are all paired (e.g., left and right anterior hook), we used the average measurement for all pairs of anterior hooks, posterior hooks, and spicules.

We make special note that in these pentastomes the anterior hooks were smaller (e.g., anterior traits were 11–50% smaller in size to that of a corresponding posterior trait) and sharper than the posterior hooks and, as such, we found these were much more difficult to measure accurately, especially when using images from a single focal plane. Hence, we a priori expected anterior measures to contain more error. Accordingly, we had more missing data for the anterior hook measures due to difficulty in orienting the smaller hooks on the slides. These issues are compounded in males because they are smaller and have smaller structures than do females.

Analyses

Pentastome data in Kelehear et al. (2011) from host species *R. marina* and *H. frenatus* were incorporated where possible to test for more-global patterns and draw more robust and generalized conclusions about sources of morphological variation in pentastomes. For simplicity, we abbreviate the host names in the presentations of the statistical analyses: HETU, *H. turcicus*; ANCA, *A. carolinensis*; RHMA, *R. marina*; and HEFR, *H. frenatus*. Also, in the tests below, we analyzed male and female morphometrics separately due to their sexual dimorphism (Ali and Riley, 1983; Kelehear et al., 2011).

2D plot of female posterior hook AB and BC: Historically, a 2D plot of female posterior BC by AB hook measurements was used to view clusters, which in turn was used to delimit species (Ali et al., 1981; Riley, 1986). However, Kelehear et al. (2011) showed that in these 2D plots, discrete clusters disappeared after accounting for pentastome body size. They concluded that the clusters were likely driven by pentastome development. This important finding suggested that body size should be accounted for in downstream analyses of hook measurements. To examine the generality of the Kelehear et al. (2011) results, we repeated their analysis on our ANCA and HETU samples. A 2D plot was constructed with raw values and then repeated to account for body size (i.e., the plot was made with the residuals of BC and AB hook measurements based on linear regressions with body length).

At this point, we note that females of *R. indica* can be gravid in distinct instar developmental stages inside their final host (Ali and Riley, 1983). To determine the developmental stage of parasites in our combined dataset, we took the instar specific hook measurements (mean, min, and max) from the experimental infections of Ali and Riley (1983) (see their table 5) and superimposed their measurements onto our data and that of Kelehear et al. (2011). We found that the clusters in the 2D-plot of AB by BC posterior hook measurements corresponded to the seventh, eighth, and ninth instar stages of female *R. indica* (*R. frenatus* [sic]) (see Results). By delimiting instar stage we could provide a more explicit and discrete means of accounting for development (e.g., tests for stage-specific patterns) while also allowing us to use body size as a separate covariate. With the exception of the principal components analyses, downstream analyses categorized female individuals into instar stages based on cluster cutoffs (see Results). Males are mature only in a single instar stage (Ali and Riley, 1983) and, thus, are not expected to form clusters based on the 2D plot when examining worms from a single host species. Indeed, this is what we observed (data not shown; see also fig. 5 in Kelehear et al., 2011); hence, males were not subdivided into instar stages.

Trait relationships: Prior studies have treated the different hook measures as independent traits, so one of our main objectives was to test for possible relationships among the 6-hook measurements used in our study (AB and BC of anterior and posterior hooks and the anterior and posterior hook bluntness areas). With both the female and male data sets, we conducted a principal components analysis (PCA) to determine whether there were latent relationships among the hook measures. PCA was conducted with the psych package v1.7.2 in R v3.3.3 and (Revelle, 2017; R Core Team, 2017, respectively) using Varimax rotation. Factor loadings of $>|0.5|$ were considered significant given our sample sizes (Hair et al., 1998). The female PCA data set consisted of $n = 196$ total pentastomes ($n = 152$ from 34 HETU, $n = 23$ from 4 ANCA, $n = 15$ from 5 RHMA, and $n = 6$ from 4 HEFR). The male PCA data set consisted of $n = 109$ total pentastomes ($n = 91$ from 28 HETU, $n = 3$ from 2 ANCA, $n = 7$ from 5 RHMA, and $n = 8$ from 4 HEFR). Using the male data set, a simple linear regression model was used to test for a correlation between spicule length and width. Based on the results of the PCA (see Results), downstream analyses on hook measurements used a summated score of the AB and BC measures of the posterior hooks. For simplicity, we refer to this summated score as 'hook size.'

Testing factors that could influence morphology: We tested whether 2 host factors (SVL and host sex) and a context-specific factor (i.e., parasite density-dependence as a function of the infection intensity) were associated with morphological traits to

ascertain the potential for environmental-induced morphological variation. These tests were conducted separately for the eighth and ninth instar females and for males. Tests were conducted with the R packages *lme4* v1.1.12 and *lmerTest* v2.0.36 (Bates et al., 2015; Kuznetsova et al., 2017) using linear mixed-effect models where *P*-values were calculated according to the Satterthwaite approximation. Pentastome body length was the dependent variable and host sex, host SVL, and pentastome intensity were the main effects. The random effects were sampling location and individual host nested within location. All 2-way interactions of main effects were tested; if non-significant, they were pooled.

Next, we used hook size as the dependent variable and again conducted tests separately for eighth and ninth instar females and for males. Main and random effects were the same as above, but we also included pentastome body length as a covariate. The inclusion of body length in these models was to control for any additional growth differences that may occur independently of instar stage. In males we repeated the same analyses but used either spicule length or width as dependent variables.

The female eighth instar data set consisted of n = 59 total pentastomes from 24 HETU while the ninth instar dataset consisted of n = 110 total pentastomes from 22 HETU. The male data set consisted of n = 105 total pentastomes from 30 HETU.

Testing host species as a factor: For the analyses testing for a host species effect on female traits, only ninth instar female worms were used (n = 110 from 22 HETU, n = 21 from 4 ANCA, n = 4 from 3 HEFR, and n = 8 from 4 RHMA) because there were too few samples from some host species at the eighth instar category (e.g., n = 2 from ANCA and n = 2 from HEFR). We first tested for a host species effect on pentastome body size, where host species was the main effect and individual host ID was the random effect. To test for an effect on hook size, host species and pentastome body size were used as the main effects with host ID as the random effect. The same models as above were used in male worms but with additional tests for effects on spicule length or width (n = 105 from 30 HETU, n = 5 from 3 ANCA, n = 8 from 4 HEFR, and n = 10 from 5 RHMA). We did not incorporate the variables of host sex, SVL, or pentastome intensity in the above analyses because sample sizes were too small from some host species or the data were not available for HEFR and RHMA from the study Kelehear et al. (2011). As we did not find any consistent effects of host sex, SVL, or pentastome intensity on hook size with the HETU samples alone (see Results), we do not expect that the analyses of host species effects are unduly affected by the exclusion of these variables.

RESULTS

Sampling and DNA data

From Ingleside, 37 of 68 (54.4% prevalence) geckos were infected with a mean intensity (number of pentastomes per infected host) of 9.16 (range 1–62), and from Port Aransas, 36 of 50 (72%) geckos were infected with mean intensity of 11.53 (range 1–107). Of the 88 geckos sampled from Metairie, Louisiana in 2012, 52 (59.1%) were infected with a mean intensity of 7.21 (range 1–43). Five of the 22 (22.7%) anoles sampled from Metairie, Louisiana in 2012 were infected with a mean intensity of 6.6 (range 1–15). We note that 5 of these 22 anoles, one of which was infected, were collected from a house in River Ridge, Louisiana (approximately 8.7 km from the Metairie location), but we combined them with the Metairie samples due to the proximity and small sample size.

All 24 DNA sequences, which included 8 from green anoles from Louisiana and 16 from geckos (8 from Texas and 8 from Louisiana), matched 100% of 604 base pairs of pentastomes collected in Australia (JF975594.1, Kelehear et al., 2011). Along with the genetic data, the club-shaped base of the male spicules (an important taxonomic trait; Ali et al., 1985) matched between worms from green anole and Mediterranean gecko hosts (Suppl. Data, Fig. S1; n = 105 male pentastomes from geckos, n = 5 male pentastomes from anoles examined) and, importantly, to previous reports of *R. indica* (see plate 2C in Ali and Riley, 1983; fig. 4 in Kelehear et al., 2011; fig. 4 in Barton and Riley, 2004). Based on the above evidence, we identified the pentastomes in our study as *R. indica*.

Analyses

2D plot of female posterior hook AB and BC: The 2D plot of the female posterior AB and BC hook measures showed that HETU and ANCA samples (combined or considered independently) grouped into 3 clusters (Fig. S2A). After allometrically correcting for body length, the distinction among the clusters disappeared (Fig. S2B). We confirmed that several developmental stages of females were present in our samples by superimposing data from the experimental gecko infections of Ali and Riley (1983) onto our data and that of Kelehear et al. (2011) (Fig. 1). These clusters and the data of Ali and Riley (1983) enabled us to classify female samples into 3 distinct instar stages (seventh through ninth) which in turn provided a more explicit control variable for development in subsequent analyses. We demarcated instar stages using the posterior AB measurements as follows: seventh instar, less than 138 μm; eighth instar, inclusive measurements 138 μm through 233 μm; ninth instar, measurements greater than 233 μm (Fig. 1). We recognize these cutoffs are somewhat subjective when data from all host species are combined, but they are relatively unambiguous when only considering worms from a single host species. When considering samples from all host species, ambiguous cutoffs would be expected if indeed there were host species effects on these measures (we address host effects below).

Trait relationships: The PCA results from both the female and male data sets showed that 5 of the hook measurements loaded very highly and significantly onto a single factor and that the area of the anterior hook tip loads by itself on a second factor (Table I). Validation of the factors was evidenced by the congruent results of the independent male and female data sets (Hair et al., 1998). With the exception of anterior hook bluntness, these results indicated that the traditional hook measurements highly covary and, as such, treating each as independent runs the risk of pseudoreplicating a single underlying trait. Therefore, to avoid pseudoreplication and considering other issues detailed below, we decided to focus our subsequent analyses on a summated score (i.e., average) of the AB and BC measures of the posterior hooks (i.e., hook size). We wanted to include these traits because the AB and BC posterior hook measures have traditionally been used in 2D plots to delimit species (Ali et al., 1981; Ali and Riley, 1983; Riley, 1986) and instar developmental stages (Ali and Riley, 1983). Using summated scores is an appropriate way to summarize correlated variables of the same trait, i.e., an aspect of hook size in these pentastomes, while also helping to reduce

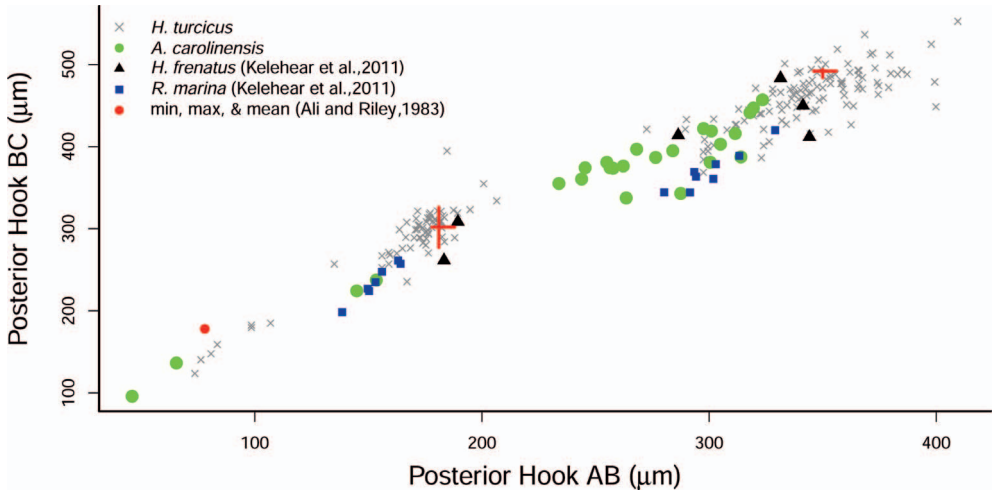

Figure 1. Posterior hook BC by AB measurements of female *Raillietiella indica* from *Anolis carolinensis*, *Hemidactylus frenatus*, *Hemidactylus turcicus*, and *Rhinella marina*. The red crosses represent the min, max, and means (the crux) of females in the eighth and ninth instar stages and the red dot represents a single seventh instar specimen, all from the experimental gecko infections of Ali and Riley (1983). Qualitatively (visually in this graph) and statistically (see Fig. 3), pentastomes from *A. carolinensis* and *R. marina* tend to have smaller hook sizes relative to those infecting gecko hosts.

measurement error (Hair et al., 1998). Moreover, a summated score is comparable across studies whereas standardized factor scores are only comparable within data sets. Although the anterior hook bluntness could represent an independent trait, we also had the most difficulty in measuring this trait. Hence, its loading on a separate factor could indicate greater measurement error in this variable. As we noted in the methods, we had more missing data for anterior hooks in general. By excluding the anterior hook AB and BC measurements, we were able to include more samples into our analyses. Lastly, although the posterior hook bluntness also loaded highly onto the first factor (Table I), we did not include it in the summated score because it is a measure of area as opposed to a linear measure. Simply put, a summated score of the posterior hook AB and BC measures should reflect overall hook size while reducing error.

Table I. PCA results on hook measurements: variable factor loadings, factor eigenvalues, and proportion of total variance explained by each factor from the Varimax rotated correlation matrix of all sampled worms (see main text for sample sizes). In the female dataset, n = 152 from 34 *Hemidactylus turcicus* (HETU), n = 23 from 4 *Anolis carolinensis* (ANCA), n = 15 from 5 *Rhinella marina* (RHMA), and n = 6 from 4 *Hemidactylus frenatus* (HEFR). In the male dataset, n = 91 from 28 HETU, n = 3 from 2 ANCA, n = 7 from 5 RHMA, and n = 8 from 4 HEFR.

	Varimax rotated loading matrix	
Hook measurements	Factor 1	Factor 2
A. Female		
Posterior hook AB (µm)	0.97	0.08
Posterior hook BC (µm)	0.97	0.01
Anterior hook AB (µm)	0.89	0.24
Anterior hook BC (µm)	0.93	0.16
Mean area of the posterior hook (µm^2)	0.94	0.04
Mean area of the anterior hook (µm^2)	0.09	0.99
Rotated eigenvalues	4.43	1.07
Percent total variance explained	0.74	0.18
B. Male		
Posterior hook AB (µm)	0.78	−0.32
Posterior hook BC (µm)	0.89	−0.20
Anterior hook AB (µm)	0.67	0.16
Anterior hook BC (µm)	0.81	0.22
Mean area of the posterior hook (µm^2)	0.79	−0.25
Mean area of the anterior hook (µm^2)	−0.04	0.92
Rotated eigenvalues	3.13	1.12
Percent total variance explained	0.52	0.19

Spicule length and width of males were correlated ($F_{1,107}$ = 13.45, $P < 0.001$, $r^2 = 0.10$). However, because of the low r^2, we analyzed spicule length and width separately.

Factors that could influence morphology: No interactions or main effects were significant indicators of body length of eighth instar females; however, host sex was marginally non-significant where pentastomes in female HETU were larger than those in males ($F_{1,20.70} = 4.29$, $P = 0.051$; Suppl. Data, Table S1A). No interactions were significant with body length of ninth instar females. Pentastome intensity was negatively related to body length of ninth instar females ($F_{1,14.06} = 7.51$, $P = 0.02$; Table S1B). For male body length, no interactions were significant, but there was a negative relationship with host SVL ($F_{1,17.34} = 7.13$, $P = 0.02$; Table S1C). Considering the same hypothesis (i.e., statistical model) was conducted for three groups (female eighth and ninth instars and males), no variables were significant with a Bonferroni correction (adjusted P-value = 0.0167).

No interactions or any of the potential environmental variables (host sex, SVL, or parasite intensity) were associated with hook size in the eighth or ninth instar stage of female pentastomes (Table S2). Only the covariate body length had a positive association with hook size in the ninth instar females ($F_{1,102.68} = 13.21$, $P < 0.001$; Table S2B). For male worms, there was a significant interaction between pentastome intensity and host sex ($F_{1,13.58} = 5.18$, $P = 0.04$; Table S2C) where intensity was positively correlated with hook size in female hosts but negatively correlated in male hosts. Again considering multiple testing, this latter interaction was not significant with a Bonferroni correction (adjusted P-value = 0.0167) and upon excluding the interaction from the model, host sex was no longer significant ($F_{1,15.71} = 0.34$, $P = 0.57$).

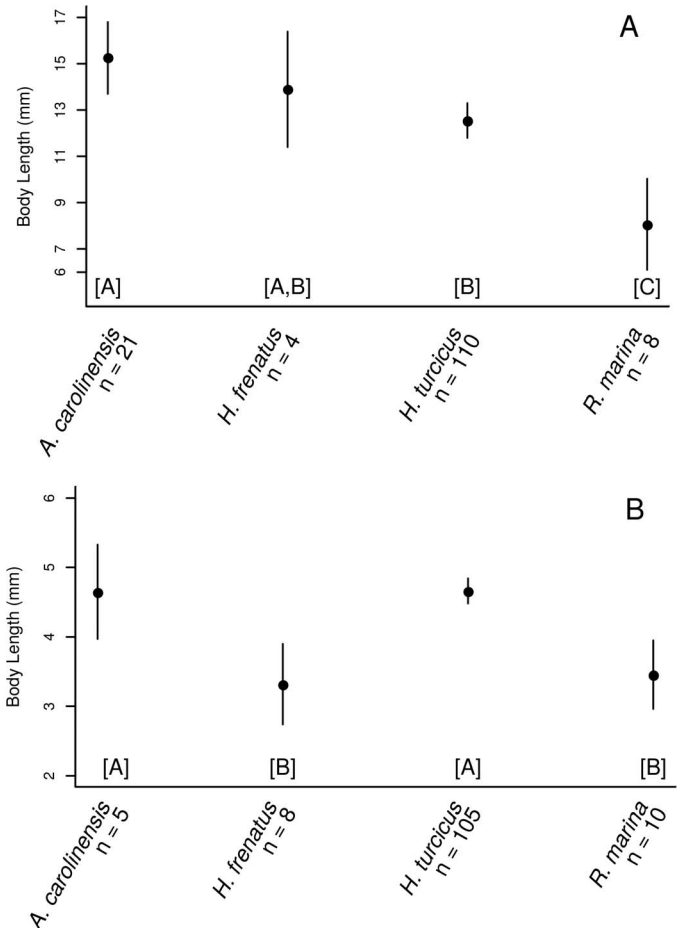

Figure 2. Pairwise comparisons of pentastome body length between host species in (**A**) ninth instar females and (**B**) males. Black bars indicate 95% confidence intervals. Letters denote significant differences in the post hoc pairwise comparisons (all shown differences are after a Bonferroni correction, $P < 0.0083$).

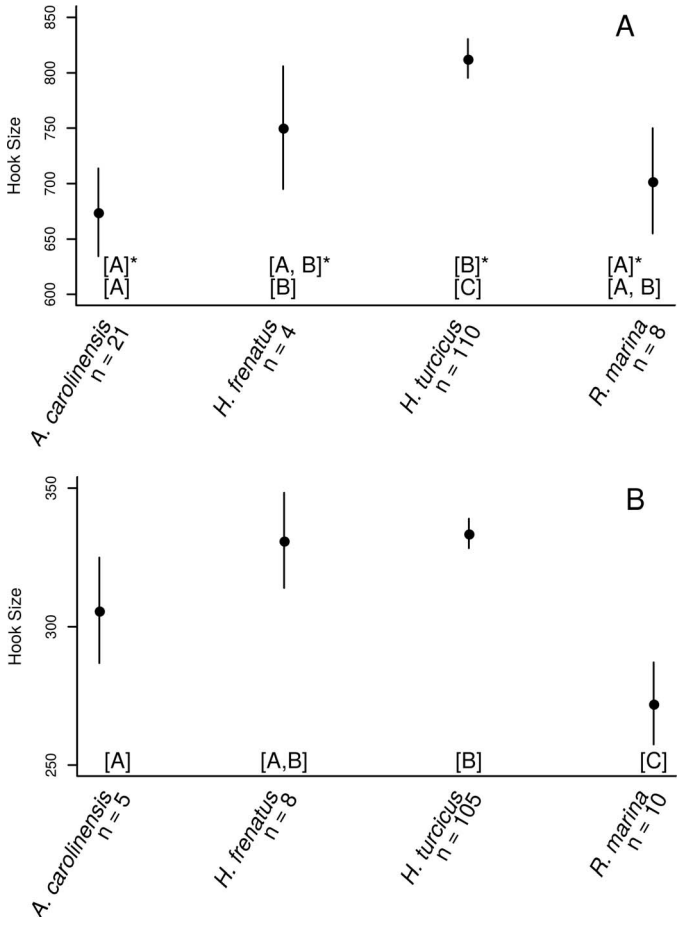

Figure 3. Pairwise comparisons of pentastome hook size between host species in (**A**) ninth instar females and (**B**) males. Black bars indicate 95% confidence intervals. Letters denote significant differences in post hoc pairwise comparisons. In (A), plain letters show significant differences at $P < 0.05$ whereas differences based on a Bonferroni correction ($P < 0.0083$) are shown with asterisks. In (B), all shown differences are after a Bonferroni correction, $P < 0.0083$.

For spicule length, the final model showed a negative relationship with pentastome intensity even when controlling for the covariate body length, which itself was positively correlated to spicule length ($F_{1,4.79} = 11.10$, $P = 0.02$; $F_{1,96.52} = 4.85$, $P = 0.03$, respectively; Table S3A). A negative relationship between intensity and spicule width was marginally non-significant ($F_{1,8.19} = 4.64$, $P = 0.06$; Table S3B).

Testing host species as a factor: Female body length at the ninth instar stage was significantly different among host species ($F_{3,30.29} = 11.035$, $P < 0.001$; Table S4A). In post hoc pairwise comparisons (difflsmeans function from the *lmerTest* v2.0.36 R package; Kuznetsova et al., 2017), pentastome body size decreased significantly from ANCA to HETU to RHMA (Fig. 2A; Table S4B). Body length of pentastomes in HEFR (n = 4) was similar to those in ANCA and HETU (Fig. 2A; Table S4B). In male pentastomes, host species also had a significant effect on body length ($F_{3,51.11} = 12.209$, $P = < 0.001$; Table S4C), and post hoc pairwise comparisons showed male pentastomes from ANCA and HETU were larger than those from HEFR and RHMA (Fig. 2B; Table S4D).

Controlling for the covariate body size (itself with a positive relationship $F_{1,135.91} = 16.528$, $P < 0.001$), hook size in ninth instar females was significantly different among host species ($F_{3,39.28} = 18.562$, $P < 0.001$; Table S5A). Post hoc pairwise comparisons showed that hook size was the largest among pentastomes in gecko hosts (Fig. 3A; Table S5B). Similarly, hook size was significantly different among host species in male pentastomes ($F_{3,54.72} = 21.4475$, $P < 0.001$; Table S5C) where again geckos tended to harbor pentastomes with the larger hooks (post hoc pairwise comparisons; Table S5D; Fig. 3B).

Spicule length was not related to host species when controlling for the covariate body length, which itself had a positive relationship to spicule length ($F_{1,122.81} = 7.43$, $P < 0.01$; Table S6A). Spicule width had a marginally non-significant association with host species ($F_{3,51.10} = 2.74$, $P = 0.053$; Table S6B).

DISCUSSION

The key findings of our study indicate that there are three critical considerations regarding raillietiellid (possibly pentastomes in general) taxonomy and the resolution of cryptogenic status for these parasites. First, the majority of hook traits were not independent for *R. indica*. Thus, for other pentastome species,

it should be determined whether hook traits can be treated as separate variables in statistical analyses. Second, we explicitly show that key hook traits vary according to instar (i.e., developmental) stage. Third, host species was associated with significant differences in morphological variation of important taxonomic traits in pentastomes, even when accounting for body length and instar stage. By attributing morphological variation to developmental stage and host species, and confirming morphological identification with genetic sequences, we determined that the pentastome *R. indica* is an invasive parasite that lacks host specificity and has spilled-over into a native host in the United States.

Variation in taxonomic traits

Species identification based on morphological traits requires knowledge of the extent of trait variation. Measurement error aside, sources of variation include investigator-induced variation during handling and specimen preparation, variation across organismal growth and development, environmental-induced variation, and underlying genetic variation. A trait will be diagnostic if between-species genetic variation exceeds within-species genetic variation. The other sources of variation create noise and could possibly lead to inaccurate reports of the number of species present in a study.

Not only are taxonomic studies of pentastomids hindered by environmental and developmental factors that affect morphological variation, but they are also affected by a lack of external structures suitable for fixation and thus identification (Riley, 1986; Kelehear et al., 2011). Metrics such as body shape, hook morphology, annulus number, and the position of the female gonopore are suitable for broad generic identification, but minute differences and often overlapping trait ranges can make specific diagnosis ambiguous (Riley, 1986). The latter problem is compounded by the fact that species descriptions often use few specimens (Riley, 1986). Thus, the range of intraspecific morphological variation is poorly understood.

To avoid specimen preparation-induced artifacts, Riley (1986) emphasized the use of rigid structures such as hooks and copulatory spicules. In particular, cluster patterns observed in 2D plots of BC by AB female posterior hook measurements have been advocated as a means for species identification (Riley, 1986). Nevertheless, Riley (1986, p. 59) noted that such plots "can only be meaningfully compared between fully adult specimens." This statement acknowledges that raillietiellid pentastomes undergo molts (i.e., female instar developmental stages 7, 8, and 9) in their definitive hosts (Ali and Riley, 1983). Indeed, plots of female BC by AB posterior hook measurements form distinct clusters based on instar stage (Fig. 1; Fig. S2; see also tables 3 and 5 in Ali and Riley, 1983). Thus, it is necessary to establish instar stage to meaningfully compare morphology among species. Yet, there is no clear way of doing this outside of controlled infections. Ali and Riley (1983) reported the percentage of fully developed eggs in the uterus was correlated with instar stage but, as noted by Kelehear et al. (2011), this is undoubtedly a tedious and time-consuming metric to obtain.

Our study improves pentastome taxonomy by illustrating an approach that can be used to determine whether variation in morphological traits is due to variation among development stages or among-species variation. Sequence data (discussed below) along with overall spicule shape were the best indicators that the native and exotic lizards were infected by a single species of pentastome. Independently confirming the results of Kelehear et al. (2011), we found that when allometrically correcting for body length, the observed clusters in the 2D-plot disappeared (Fig. S2). Based on this latter result, Kelehear et al. (2011) hypothesized that hook size was also affected by development. Here, we explicitly make the connection between the 2D-plot clusters and discrete instar developmental stages by superimposing data from the experimental infections of Ali and Riley (1983) onto our data and that of Kelehear et al. (2011). Interestingly, the experimental infection data from Ali and Riley (1983) were generated from gecko hosts (*H. frenatus* and *Cosymbotus platyurus*). The qualitative concordance among the data from the three studies is remarkable, especially when comparing data from phylogenetically similar host species (i.e., geckos). The overlay in Figure 1 clearly shows that 2D-plot clusters of female BC by AB posterior hook measurements correspond to the seventh, eighth, and ninth instar stages (Fig. S2). Nevertheless, it is also apparent in this 2D-plot that the hook morphology might vary according to host species; a result that suggests hook size variation could be influenced by environmental-induced variation.

Prior to testing if various environmental factors (including host species) were associated with hook sizes, we first tested the assumption that the six hook measurements were independent traits. Five of the 6 hook traits were highly correlated and hence loaded onto a single factor in the PCA (Table I). We speculate that anterior hook tip area could have formed its own factor because of measurement error itself. Using a composite score of the AB and BC posterior hook measurements along with the ability to delimit instar stages, we then tested for associations of various factors that might induce environmental variation in hook size.

Taking advantage of the large sample size from *H. turcicus*, we tested if host sex, host SVL, and parasite density-dependence could be factors that may induce variation in pentastome body size or hook size. Some variables were significant. For example, in ninth instar female worms, body length was negatively correlated with intensity (Table S1B); a pattern consistent with what would be expected under density-dependent growth. However, this effect would have to be instar stage-specific as we did not find a density-dependent effect on eighth instar female body length (Table S1A). In general, we did not see consistent patterns across these tests of host sex, host SVL, and parasite density-dependence on pentastome body size or hook size. So, the interpretation of results should be regarded with caution, especially considering we performed multiple tests. Some of the patterns may be real, but additional studies under more-controlled conditions are needed for confirmation. With regard to male spicules, however, we found a consistent effect of parasite intensity. Spicule length was negatively associated with parasite intensity while width was marginally so (Table S3). Hence, spicules could be influenced by intraspecific crowding effects.

In contrast to the mixed results described above, we found that pentastome body length and hook size significantly varied among the different host species and, qualitatively, patterns were similar for ninth instar females and males (Figs. 2, 3). For body length, pentastomes from ANCA and HETU were larger and those from RHMA were the smallest (Fig. 2). Hook sizes, while controlling

for body length, tended to be larger for both male and female pentastomes from gecko hosts (HETU and HEFR) and smaller from the toad (RHMA) and anole (ANCA) hosts (Fig. 3). Observed discrepancies from these general patterns could be due to small sample sizes from some host species (e.g., n = 4 for the ninth instar females from HEFR). Although we did not statistically test the seventh or eighth instar females, qualitatively, the 2D-plot of BC by AB hook measurements showed that hook size in these stages is also smaller in ANCA and RHMA compared to the collective samples from gecko hosts (collected across 3 independent studies; see Fig. 1). Interestingly, despite worms growing to a much larger size in green anoles (Fig. 2), hook sizes were smaller for a given body size in ANCA (Fig. 3). This latter result may suggest that different allometric growth patterns for hook size are induced when developing in different host species; a result also seen in Kelehear et al. (2011) for pentastomes from RHMA. The above patterns suggest that for a given trait, host species affect male and female worms similarly, but that patterns may differ among the traits themselves. In particular, spicule measures were largely robust to host species of origin (Table S6). This last result may reinforce the use of spicule measurements as a raillietiellid species diagnostic trait (Riley, 1986) but, as noted above, their size could be subject to density-dependent effects. Therefore, the overall shape (see Ali et al., 1985), which was used in our identification, may be a more reliable character than size.

On the whole, the developmental- and host-induced variation we and Kelehear et al. (2011) observed may explain, in part, much of the taxonomic confusion as presented by Poore (2012). We suspect the difficulty in assessing different instar stages of mature female raillietiellids is what led to the initial description of *R. frenatus* [sic] as a different species from *R. indica*. As discussed in Kelehear et al. (2011), it is very plausible that the species description of *R. frenatus* [sic] (Ali et al., 1981) was based on a later instar stage of *R. indica*.

Sequence data

Based on the 100% identity in the *CO1* sequences (n = 16 from HETU, n = 8 from ANCA), as well as the similarities in spicule shape (Fig. S1), we are confident that the pentastomes we collected from Mediterranean gecko and green anole hosts within the southern United States are the same species as those from Australia (n = 26 from RHMA and n = 8 from HEFR; Kelehear et al., 2011). A recent study on an invasive pentastome, *Raillietiella orientalis*, into Florida via Burmese pythons found only a single *CO1* haplotype (n = 3 from pythons, n = 7 from native snakes; Miller et al., 2018). In comparison to 3 haplotypes reported from Australian snakes (n = 12; Kelehear et al., 2014), there was 1.2% divergence from the most common haplotype (n = 10; see haplotype network in fig. 7 of Miller et al., 2018). The lack of variation among mitochondrial haplotypes for both *R. indica* and *R. orientalis* in the United States could have resulted from recent introductions via strong founder effects. Nonetheless, the absence of variation among *R. indica* from the United States (our study), Australia (Kelehear et al. 2011), and Panama (Kelehear et al., 2015) is an unexpected result given the generally high mutation rate of mtDNA (Ballard and Whitlock, 2004). Some possible explanations for the lack of variation include that the mtDNA of pentastomes has a slow evolutionary rate (e.g., low metabolic rate in sharks is associated with low mitochondrial substitution rates; Martin, 1999) or that there has been a recent and strong selective sweep in the mitochondria for this species. Another possibility is that *CO1* has been incorporated into the nuclear genome, which will typically have a lower mutation rate relative to the mitochondria (Ballard and Whitlock, 2004). There is no evidence that the amplified locus is a pseudogene, as mutations likely would have accumulated. Also, the *CO1* sequence translates with no premature stop codons according to the invertebrate mtDNA code 5, suggesting the amplified locus is functional (though mutations in flanking regions remain a possibility). Regardless of the cause for the lack of variation, the main caveat is that the lack of variation precludes conclusive inferences on colonization history. As such, it will be necessary to obtain more genetic markers to aid in future studies on taxonomy or within-species population history.

Range, host expansion, and resolving the cryptogenic status

To our knowledge, the first report of a species of *Raillietiella* in the continental United States was in 1981 from southern Texas. Pence and Selcer (1988) documented *R. indica* (*R. frenatus* [sic]) from *H. turcicus* in Edinburg, Texas. Since then, *Raillietiella teagueselfi*, which has a distinctive spicule shape compared to *R. indica* (see fig. 2 of Riley et al., 1988), was described from *H. turcicus* in Houston, Texas. However, until our study neither of these species has been reported in native lizards in the southern United States. Most recently, Miller et al. (2018) reported the spillover of *R. orientalis* from Burmese pythons into native snakes in Florida. We note a report of *Raillietiella bicaudata* in North America was not considered because it may not be a valid species (see Miller et al., 2018).

Here, we report a range expansion of *R. indica* into Port Aransas, Texas and surrounding areas by 2012 (Caballero et al., 2015) as well as into Metairie, Louisiana (part of the metropolitan area of New Orleans). The range expansion into Louisiana may have occurred within the last 20 yr because Metairie survey collections from 1997–1999 did not reveal any pentastome infections in *H. turcicus* (n = 42) or *A. carolinensis* (n = 11) (Criscione, 2000; Criscione and Font, 2001c). Moreover, an additional 184 geckos sampled from 5 additional locations in southeastern Louisiana in 1998 also did not have pentastome infections (Criscione and Font, 2001c). In 2008, a single-sampled Mediterranean gecko from the Metairie site was infected with *R. indica* (C.D.C., unpubl. data). Indeed, this finding prompted the additional 2012 surveys in Metairie from both the exotic Mediterranean gecko and the native green anoles on which our current study is based. Importantly, this study documents a host expansion into the green anole in its native range.

There could be three possible routes of pentastome colonization in Louisiana. First, New Orleans is a major world port, offering a path for infected geckos or possible intermediate hosts (e.g., cockroaches) to be transported into the city from around the globe. However, *H. turcicus* has been reported in the New Orleans area since 1949 (Etheridge, 1952), and the 1997–1999 surveys did not find any pentastome-infected geckos. Second, although anecdotal, pentastomes could have colonized the area after Hurricane Katrina in 2005. Recovery and cleanup efforts involved temporary workers from southern Texas. So, it is plausible that

infected gecko or cockroach intermediate hosts were transported from southern Texas. Third, the Cuban brown anole (*Anolis sagrei*) naturalized in New Orleans from the 1990s to early 2000s (Lever, 2003). Interestingly, *R. indica* (*R. frenatus* [sic]) has been reported from brown anoles and green anoles in Hawaii (Goldberg and Bursey, 2000; Goldberg et al., 2004). So, another plausible scenario is that invading populations of brown anoles also harbored pentastome infections.

Given the collective morphological data in our study and in Kelehear et al. (2011), we postulate that most reports of *R. frenatus* [sic] are likely *R. indica*. Poore (2012) provides an excellent discussion of taxonomy in the genus *Raillietiella* (including details of *R. hebitihamata*, *R. frenata*, and *R. indica*) and concludes that the name *R. indica* has seniority. Although we were able to resolve the morphological issues and identify the species as *R. indica*, there remains a problem in resolving the cryptogenic status.

Anthropogenic transport of host species prior to taxonomic surveys complicates identification of exotic parasite origins (Carlton, 1996). This would be especially problematic in geckos as they represent one of the most successful establishing families of alien reptiles or amphibians known (Detwiler and Criscione, 2014). Indeed, this issue has been raised before for other parasites found in the Mediterranean gecko in the southern United States. In particular, Criscione and Font (2001a) discussed how tapeworm species of the genus *Oochoristica* may have colonized new areas before many of them were ever described. In the case of *R. indica*, which appears to have a lack of host specificity, the original description was from an anuran host at the Indian Museum in Calcutta, but no specific type locality was given (Gedoelst, 1921). Most subsequent reports of *R. indica* or *R. frenatus* [sic] have occurred throughout Southeast Asia (Poore, 2012; see Kelehear et al., 2013 for a comprehensive review of host and distributional records). More-recent reports of *R. indica* include Australia (Kelehear et al., 2011), Hawaii (Barton and Riley, 2004), Brazil (Anjos et al., 2008), and Panama (Kelehear et al., 2015).

The preponderance of reports from Asia and the historical dates of reports suggest that *R. indica* is indeed an invasive parasite that now has spilled over into a native host (green anoles) in the southern United States. The spillover of *R. indica* into green anoles in their native range is important because of the potential for adverse fitness effects that pentastomes may impose on their hosts. For example, Pence and Selcer (1988) found pentastome infections were associated with a reduction in the number of oviductal eggs of *H. turcicus*. In addition, Caballero et al. (2015) found that after intense activity, the metabolic recovery time of *H. turcicus* was prolonged with increasing numbers of pentastomes, demonstrating a potential physiological cost of infection. These potential effects, along with the mounting competition that green anoles may face from the introduction of brown anoles (Campbell, 2000), give cause for concern. In addition, given the apparent lack of host specificity demonstrated by *R. indica*, there could very well be other native reptile and amphibian species infected.

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