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FAST TRACK

Origin of the parasites of an invading species, the Australian cane toad (*Bufo marinus*): are the lungworms Australian or American?

SYLVAIN DUBEY and RICHARD SHINE
School of Biological Sciences A08, University of Sydney, Sydney, NSW 2006 Australia

Abstract

Phylogeographical analyses that identify the geographical origin of parasites in invading species can clarify the parasites' potential for biological control of the invader and the risks posed by the parasite to native species. Our data on nuclear and mitochondrial genetic sequences show that the nematode lungworms (*Rhabdias* spp.) in invasive Australian populations of cane toads (*Bufo marinus*) are *Rhabdias pseudosphaerocephala*, a South American species. We did not find this lungworm species in any Australian frogs sympatric with cane toads, suggesting that the parasite does not attack Australian frogs and hence may offer potential as a biocontrol agent of the toad.

Keywords: amphibians, invasive species, mitochondrial gene, nuclear gene, parasite

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Introduction

The process of biological invasion often modifies host-parasite relationships. Invasive species may leave some or all of their ancestral (native-range) pathogens behind in the process of translocation and may be infected by novel parasites from the introduced range (Mitchell & Power 2003; Torchin *et al*. 2003). Parasite fauna in invasive species thus may consist of a mixture of native-range taxa and newly encountered taxa (Barton 1996). Distinguishing between these alternatives – that is, whether a parasite in an invasive species has come with the invader or is endemic to the introduced range – has important ramifications for two issues. First, a parasite that has accompanied the invading species may be host-specific, especially if the invader belongs to a phylogenetic lineage not represented in the introduced range. Such host specificity would facilitate use of the parasite for biological control of the invading host species, with little risk of collateral damage to native fauna (Bellows 2001). On the other hand, if the 'new' parasite is not host-specific, it may infect the native fauna also – which might lead to catastrophic effects on a nonadapted assemblage of new hosts (Hulme 2007). Neither of these issues arises if the invader's parasites are taxa from the introduced range, because in such cases the lack of host-specificity rules out use of the parasite as a biological control, and the prior distribution of the parasite means that the invaders' spread will not expose the native fauna to novel pathogens. Unfortunately, distinguishing whether the parasite of an invading species is itself translocated vs. being derived from the introduced range is far from simple. Especially in the case of internal parasites, identification is difficult because of extreme morphological simplicity, evolutionary parallelism and evolutionary convergence (Baker 1979; Kuzmin *et al*. 2007). Molecular methods can resolve this difficulty.

The most infamous amphibian 'invasive species' is the South American cane toad (*Bufo marinus*), a large (to 23.8 cm body length, 2.8 kg; Conant & Collins 1991) bufonid that is native to Central and South America but has been translocated to more than 30 countries worldwide (Zug & Zug 1979; Lever 2001). Toads were brought to Australia in 1935, in an attempt to reduce beetle-induced damage to commercial sugar-cane crops (Easteal 1981). After their introduction to northeastern Queensland in 1935, the toads spread rapidly and now occupy more than a million square kilometres of Australia (Fig. 1). Climatic models suggest that the toads may eventually extend over twice this area (Urban *et al*. 2007). The current invasion front is moving at

Correspondence: Sylvain Dubey. Fax: +61-2-93515609;
E-mail: sylvain.dubey@bio.usyd.edu.au

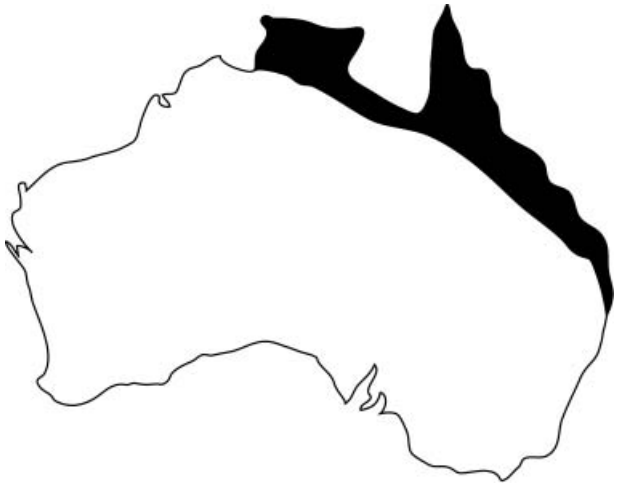

Fig. 1 The current geographical range of invasive cane toads (*Bufo marinus*) in Australia, as at mid-2008.

almost 60 km per annum, much faster than in earlier years (Phillips *et al*. 2006). Because Australia has no endemic bufonids, many frog-eating native predators naive to the toads' toxins are fatally poisoned when they attempt to eat these anurans (e.g. Burnett 1997; Griffiths & McKay 2007). Mortality due to toad ingestion also has been reported in domestic pets (Lever 2001). Hence, there is an urgent need to develop means to control cane toad populations.

The 101 adult toads that founded the Australian population were captured in Hawaii, from among the descendants of a group of toads from Puerto Rico, which in turn came from Barbados, which in turn were based on a translocation from the original Guyanan and French Guyanan populations (Easteal 1981). The history of successive translocations (involving small numbers of animals each time), and the fact that few of the original founding adults were released in Australia (Mungomery 1936; Easteal 1981), presumably reduced the probability of native-range (South American) toad parasites finding their way to Australia. In keeping with this prediction, surveys of Australian and Hawaiian cane toads have reported that these invasive populations lack many of the parasites common in native-range cane toads (Barton 1997). The macroparasite most commonly reported in Australian cane toads (but apparently lacking from Hawaiian toads) is a nematode lungworm (Barton 1996, 1998). The parasite has been tentatively identified as *Rhabdias* cf. *hylae* (Barton 1998), a species endemic to Australian frogs (Barton 1994); its presence in toads has been attributed to a host shift from Australian frogs to the invasive toad. If true, this origin would argue against the use of this nematode lungworm to control Australian cane toads and also would suggest that the parasite is unlikely to have significant impacts on the (already-adapted) Australian anuran fauna. However, because nematodes are difficult to identify on morphological criteria, we set out to verify the identity of the lung nematodes in Australian cane toads using molecular methods based on the sequencing of mitochondrial and nuclear genes.

Materials and methods

Tissue sampling and DNA extraction

Tissue samples of 90 *Rhabdias* spp. were collected in 2007 and 2008 from Australia and Brazil (Table S1, Supporting Information). We dissected 207 native Australian frogs (all road-killed) belonging to two widespread families (Hylidae and Myobatrachidae), four genera (*Cyclorana*, *Limnodynastes*, *Litoria* and *Opisthodon*), and 12 species. In addition, we dissected 92 *Bufo marinus* from Australia (27 localities) and 11 from Brazil (one locality), as well as three *B. schneideri* from Brazil. Total cellular DNA was isolated from complete *Rhabdias*. Tissues were placed in 200 μL of 5% Chelex containing 0.2 mg/mL of proteinase K, incubated overnight at 56 °C, boiled at 100 °C for 10 min and centrifuged at 13′200 g for 10 min. Then, the supernatant containing purified DNA was preleved and stored at –20 °C. GenBank Accession nos are as follows: for the mitochondrial cytochrome *b* gene (*cyt-b*), EU836833–EU836862 and for the nuclear DNA fragment, spanning the 3′ end of 18S nuclear rDNA gene, ITS region (ITS1 + 5.8S + ITS2), and 5′ end of the 28S (*ITS*), EU836863–EU836874. Additional sequences of *Rhabdias* spp. and *Strongiloides stercoralis* deposited in GenBank were also included in the analyses (Table S1, Supporting Information).

DNA amplification

Double-stranded DNA amplifications of *cyt-b* were performed with the primer pairs Rh_cytbf (5′ agt gtt caa tat att ata 3′)/Rh_cytbr (5′ ttt att agg aat agc acg 3′), and those of *ITS* with the primer pairs ITS5/ITSr (5′ caa tgc aac tcg tac agg tc 3′) and ITSf (5′ ttt cta cgg ccg atg tat ac 3′)/1500R (Kuzmin *et al*. 2007). Rh_cytbf, Rh_Rcytbf, Rcytbr, ITSr and ITSf were specifically developed for this study. Amplified products were genotyped with a 3730xl DNA analyzer using GENESCAN ANALYSIS 2.1 (Applied Biosystems). Amplification conditions included a hot start denaturation of 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 50 °C annealing temperature for 45 s, 72 °C for 90 s, and a final extension of 72 °C for 7 min. Amplified products were genotyped with a 3130xl genetic analyzer (Applied Biosystems) using GENEMAPPER version 3.7 (Applied Biosystems).

Phylogenetic analyses

The sequences were aligned by eye for *cyt-b* and by using the multiple alignment algorithm implemented in ClustalW

for *ITS* (Thompson *et al*. 1994); the latter alignments were further checked by eye. Tests were conducted on the total fragments (630 bp for *cyt-b*, 1564 bp for *ITS*); all codon positions were used. For *cyt-b*, trees were rooted using a sequence of *Strongyloides stercoralis* (Order Rhabditida, family Strongyloididae, AJ558163), and for *ITS*, the trees were unrooted due to the difficulty of finding an adequate outgroup containing the entire sequences. Maximum parsimony (MP) analyses on the *cyt-b* and *ITS* data set were performed using PAUP*4.0b10 (Swofford 2001) with 100 random additions of sequences followed by tree bisection and reconnection (TBR) branch swapping, and keeping at most 100 trees at each replicate. Branch support was estimated using 1000 bootstrap re-samples using the same heuristic settings. For maximum likelihood (ML), the models of DNA substitution were selected using hierarchical likelihood ratio tests (hLRTs) implemented in Modeltest 3.06, according to the protocol of Posada & Crandall (1998). The general time reversible model (GTR + I + G; Rodriguez *et al*. 1990) best fitted the *cyt-b* and *ITS* data sets, with base frequencies estimated from the data (*cyt-b*: A = 0.21869, C = 0.07511, G = 0.17992, T = 0.52628; *ITS*: A = 0.24721, C = 0.19691, G = 0.26825, T = 0.28763), an unequal distribution of substitution rates at variable sites (*cyt-b*: a = 0.754; *ITS*: a = 0.110) and six different substitution types [*cyt-b*: rate (A-C) = 0.08625, rate (A-G) = 14.93539, (C-T) = 10.17193, rate (A-T) = 1.33491, rate (C-G) = 0.00000, rate (G-T) = 1.0; *ITS*: rate (A-C) = 1.20360, rate (A-G) = 6.17453, (C-T) = 4.45315, rate (A-T) = 2.24686, rate (C-G) = 0.45456, rate (G-T) = 1.0].

ML heuristic searches (*cyt-b* and *ITS*) and bootstrap analyses (1000 replicates; *cyt-b* and *ITS*) were performed using PHYML (Guindon & Gascuel 2003). Bayesian analyses (BA) were performed with the GTR model for *cyt-b* and *ITS*, using MRBAYES version 3.1.2.1 (Huelsenbeck *et al*. 2001). Two independent runs were performed, each consisting of four parallel Markov chain Monte Carlo (MCMC) chains of 3 million generations for *cyt-b* and *ITS*, allowing a good convergence of the independent runs (the average standard deviation of split frequencies being lower than 0.01). Trees were sampled every 100 generations. Burn-in was assessed by comparing the mean and variance of log likelihoods, both by eye and using the program TRACER version 1.4 (Rambaut & Drummond 2003). Tree parameters reached stationarity after a burn-in period of three hundred thousand generations (for *cyt-b* and *ITS*). Optimal trees were then sampled every 100 generations to obtain the final consensus BA tree and associated posterior probabilities.

Results

Species infected by Rhabdias *spp.*

Of the 207 native frogs, 55 (28%; and including 10 of the 12 species) contained nematodes. Of cane toads collected in Australia, 45 of 92 (49%) were parasitized. All samples of *Bufo marinus* from eastern Queensland (Townsville area) contained lungworms, whereas this was true of only 60% of specimens from Borroloola (eastern Northern Territory), and 43% of toads from the Darwin area, close to the front of the toad invasion.

Cytochrome-b

We found 30 different haplotypes of 630 bp within the 90 analyzed samples, including 215 variable sites, of which 131 were parsimony informative. We did not find any insertions or deletions. Because the three phylogenetic methods yielded identical main branches, we showed the relationship between haplotypes only for the ML analysis (Fig. 2a).

Seven major lineages were well supported by bootstrap values. One of these lineages (Lineage I in Fig. 2) included parasites hosted by *B. marinus* from Brazil (identified as *Rhabdias pseudosphaerocephala* by Kuzmin *et al*. 2007), as well as those from Australia, and by *B. schneideri* also from Brazil (Table S1, Supplementary material). A second lineage (II) included parasites only hosted by Brazilian *B. schneideri*. Lineages III to VII included parasites hosted only by native Australian frogs; we are uncertain of the most appropriate nomenclature, but it seems likely that most of these animals have heretofore been included under the name *Rhabdias* cf. *hylae.*

The Kimura-2-Parameter genetic distances (K2P, Kimura 1980) between lineages varied from 2.2% (III–IV) to 14.5% (II–VII). The K2P between Lineage I (Hosts: *B. marinus* and *B. schneideri*) and lineages III to VII (Hosts: native Australian frogs) varied from 11.1% to 13.6%, and between lineage III to VII from 2.2% to 10.1%. In addition, the nucleotide diversity tended to be lower within Australian *R. pseudosphaerocephala* samples (0.0002) than within Brazilian specimens (0.0077).

ITS

Within the 107 analyzed samples (90 from our study; plus 17 from the study of Kuzmin *et al*. 2007), we found 16 different alleles of 1514 bp, showing 78 variable sites of which 45 were parsimony informative. Three sites on 1564 showed insertions or deletions. As the three methods for phylogenetic reconstruction yielded identical arrangements of the main branches, we show the relationship between haplotypes for the ML analysis only (Fig. 2b). The results obtained were congruent with those of *cyt-b*. Consequently, we have used the same numbering of lineages (e.g. I to VII) in Fig. 2 parts A and B. The sole incongruence occurred between the closely related mitochondrial Lineages IV and V, which shared a similar nuclear allele (IV–V_A1). Additional lineages (VIII–IX) included sequences from Kuzmin *et al*.

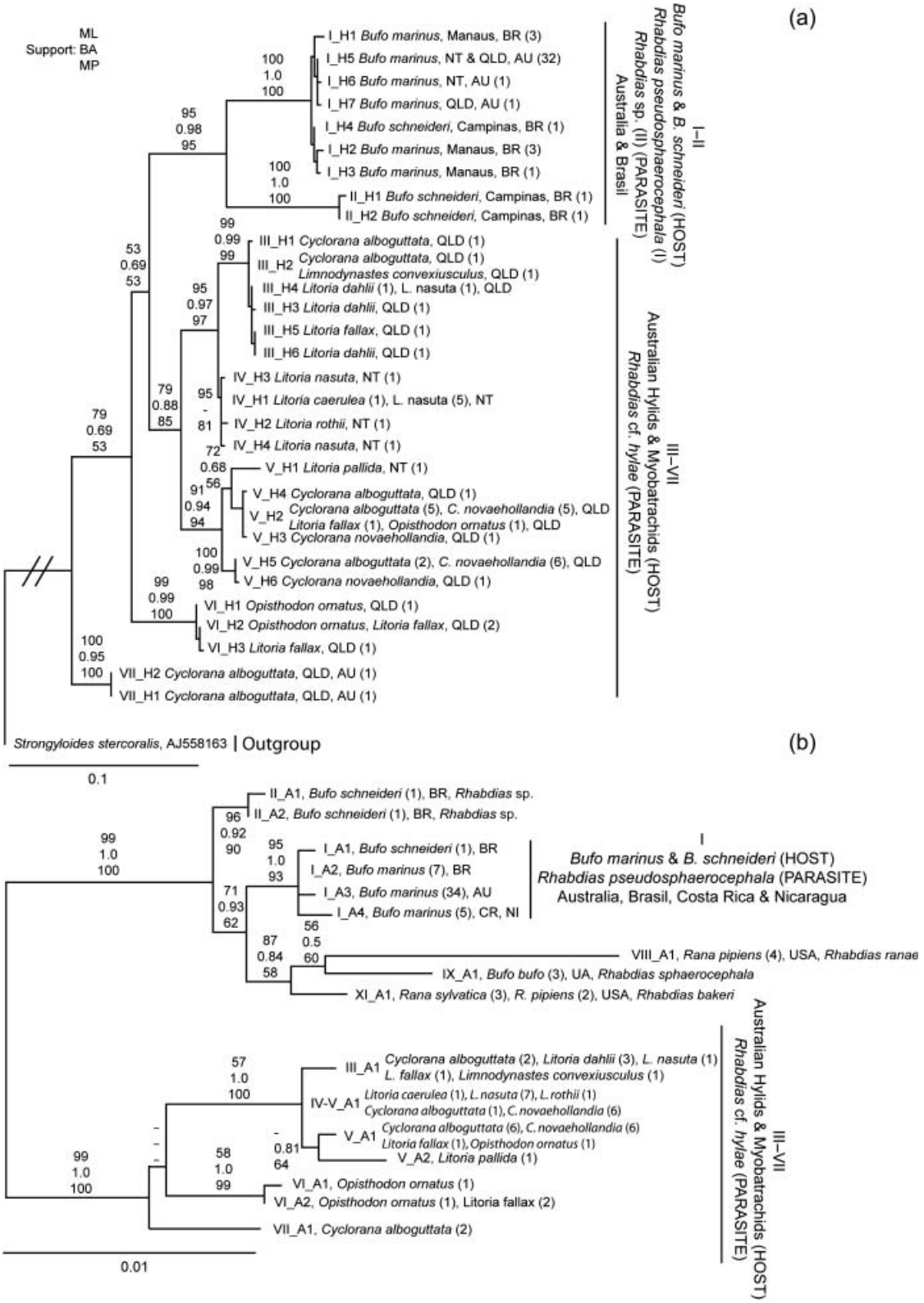

Fig. 2 Phylogeny of nematode parasites found within the lungs of Australian and South American anuran amphibians. We analyzed 630 bp of mitochondrial *cyt-b* (a) and of 1564 bp of nuclear *ITS* fragment (b; unrooted tree) with maximum likelihood (ML). Values in branches show indices of support for the major branches for ML analyses, Bayesian posterior probabilities (BA) and maximum parsimony analyses (MP). Codes are as in Table S1, Supporting Information.

(2007) of *Rhabdias ranae* (hosted by *Rana pipiens*, USA, VIII), *R. sphaerocephala* (hosted by *B. bufo*, Russia, IX), and *Rhabdias bakeri* (hosted by *Rana sylvatica* and *R. pipiens*, USA, X).

Critically, all of the samples of *Rhabdias* hosted by cane toads in Central America (Kuzmin *et al*. 2007) and South America, as well as in Australia (our study), clustered together (Lineage I), and were well differentiated from parasites hosted by native Australian frogs (Lineages II–VII). The K2P between lineages varied from 0.1% (III–'IV–V') to 3.2% (V–VIII). The K2P between Lineage I (hosts: cane toads and *B. schneideri*) and Lineages III to VII (hosts: native Australian frogs) ranged from 2.1% to 2.3%, and between Clade III to VII from 0.1% to 1.1%. Overall, the mean K2P observed between the species *Rhabdias bakeri*, *R. ranae*, *R. sphaerocephala*, and *R. pseudosphaerocephala* ranged from 0.7 to 1.5%.

Discussion

Contrary to established wisdom (Barton 1998), the nematodes in lungs of Australian cane toads are *Rhabdias pseudosphaerocephala*, a South American species (Fig. 2). Australian frogs also contain *Rhabdias* species, but not (at least in our sample) *R. pseudosphaerocephala*. Clearly, cane toads brought the lungworm with them from South America, despite a complex history of successive translocations. In previous work, the parasites of Australian toads have been identified as a species endemic to Australian native frogs (*Rhabdias* cf. *hylae*) (Barton 1998). The misidentification of the toad nematodes as *R.* cf. *hylae* was based on (i) their morphological similarity to the nematodes often found in lungs of local frogs; (ii) the lack of nematodes in Hawaiian cane toads (Speare 1990; Barton 1997; F. Kraus, personal communication.; S. Marr, personal communication.); and (iii) the translocation history. In particular, it has been suggested that the initial releases did not involve translocated toads, but only their (presumably uninfected) progeny (e.g. Easteal 1981; Barton 1997).

These lines of evidence proved misleading because: (i) morphology offers a poor guide to species identification in these animals, due to widespread evolutionary conservatism and parallelism and convergence (as in many parasites, e.g. Gong *et al*. 2006; Nadler *et al*. 2006); (ii) parasite prevalence varies through space and time, so that intensive sampling may be needed to establish absence (e.g. Readel *et al*. 2006; Batchelor *et al*. 2008; Morgan *et al*. 2008); and (iii) contrary to established wisdom, the introduction to Australia provided many opportunities for parasite transfer. First, adult toads as well as their progeny were released into waterbodies: the 1935 Annual Report of the Bureau of Sugar Experiment Stations notes that 'In addition to the distribution of toadlets a few of the originally imported toads have been liberated in nearby lagoons' (p. 53). Second, eggs, tadpoles and metamorphlings were kept with adult toads in the enclosure at Meringa Experimental Station (Mungomery 1936, p. 72) and thus could have been infected prior to release. Infective *Rhabdias* larvae can penetrate tadpoles and persist until the lungs form after metamorphosis (Baker 1979). Hence, the colonisation of Australia by *R. pseudosphaerocephala* is not surprising.

The loss of genetic diversity observed between the source population of cane toads in South America and the Australian invasive population (Slade & Moritz 1998) is mirrored in the toad's nematodes. From our results, the *cyt-b* nucleotide diversity of Australian lungworms (0.0002) was about 40 times lower than that of the Brazilian conspecifics (0.0077), presumably reflecting genetic bottlenecks during the translocation of small numbers of infected founding animals. Another major pattern in our results–the lower infection rate of toads at the invasion front compared to longer-established toad populations–may be a consequence of the rapid rate of progress of toads at the invasion front (Phillips *et al*. 2006). If infected toads are slower than uninfected ones (as shown by Kelehear 2007), we would expect the parasite front to lag behind the main toad invasion front.

Our molecular phylogenetic analyses (Fig. 2) suggest that Australian frogs are parasitized by several species of nematodes, all previously allocated to the species *Rhabdias* cf. *hylae* (Barton 1998). The genetic distances between native Australian nematode lineages (*ITS* K2P: up to 1.1%) were similar to those that we documented between recognised species (i.e. *R. bakeri*, *R. ranae*, *R. sphaerocephala*, and *R. pseudosphaerocephala*: ITS mean K2P: 0.7–1.5%). The analyses based on *cyt-b* revealed the same pattern: genetic distances (K2P) between native Australian lineages (up to 10.1%) were similar to the genetic distances between nematodes hosted by cane toads and those hosted by native Australian anurans (from 11.1% to 13.6%). More thorough sampling is needed to clarify the taxonomy of this group. Importantly, we did not find any native Australian nematodes in toads, nor any toad nematodes in native frogs. The lack of cross-over is striking, given 50 years of sympatry between toads and frogs in our primary sampling region (Townsville area).

The host specificity of nematode Lineage 1 (and the substantial genetic divergence between lineages) suggests that the cane toad nematode either (i) do not successfully infect native frogs; or (ii) kill any infected native frog so rapidly that it is not available to be sampled. We urgently need further studies to distinguish between these two alternatives. If *R. pseudosphaerocephala* is indeed host-specific to toads, it may have potential in the biological control of cane toads. Free-ranging cane toads in Australia are often heavily infected (sometimes, > 200 lungworms per toad: Barton 1998) and, if so, exhibit lowered hematocrit (presumably because the worms feed on erythrocytes: Colam 1971; Barton 1996). Metamorph cane toads infected with lungworms in the laboratory exhibit reduced rates of

survival and growth (Kelehear 2007). These parasites are absent from invasion-front toad populations in Australia, a situation that may contribute to the high abundance and large size of cane toads at the invasion front (Lever 2001; Kelehear 2007). Therefore, translocation of parasites to the invasion 'frontline' might depress toad numbers and growth rates.

An alternative interpretation of the host specificity of *R. pseudosphaerocephala* (Fig. 2) is more worrying. This parasite may attack frogs as well as toads but kill frogs so rapidly that our sampling did not detect infected animals. This scenario would suggest a hitherto-unsuspected ecological impact of cane toad invasion. We note, however, that population densities of native frogs appear to be largely unaffected by the arrival of cane toads (Greenlees *et al*. 2006; Grigg *et al*. 2006; Crossland *et al*. 2008), an observation militating against this hypothesis. Previous work in this lungworm's native range also suggests host specificity (see Kuzmin *et al*. 2007). Nevertheless, additional tests are needed to check that *R. pseudosphaerocephala* does not pose a danger to nonbufonid anurans. Such tests could simply expose metamorph frogs to infective *Rhabdias* larvae and document the consequences in terms of parasite uptake and frog viability. We also need more extensive sampling, especially across the large geographical range of *Bufo marinus*, to clarify parasite phylogeography and virulence. Only with a clear understanding of host–parasite relationships can we can we confident that we will not repeat the biocontrol mistake of the 1930's when cane toads were brought to Australia to save the sugar cane crop.

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The research interests of the authors concerns the interface between evolution and ecology in reptiles and amphibians, as well as their conservation.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1 Host species (genus abbreviation: B., *Bufo*; C., *Cyclorana*; Lim, *Limnodynastes*; L., *Litoria*; O., *Opisthodon*; R., *Rana*), parasite species, collection code, location (AU: Australia; BR: Brazil; CR: Costa Rica; NI: Nicaragua; USA: United States of America; NT: Northern Territory, AU; QLD: Queensland, AU), nuclear allele (*ITS*) and mitochondrial haplotype (*cyt-b*).

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