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SHORT COMMUNICATION

Evidence for gene flow in parasitic nematodes between two host species of shrews

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Abstract

We describe the genetic structure of populations of the intestinal nematode *Longistriata caudabullata* (Trichostrongyloidea: Heligmosomidae), a common parasite of short-tailed shrews (genus *Blarina*, Insectivora: Soricidae). Parasites and hosts were collected from a transect across a contact zone between two species of hosts, *Blarina brevicauda* and *B. hylophaga*, in central North America. An 800-base pairs (bp) fragment of the ND4 mitochondrial DNA (mtDNA) gene was sequenced for 28 worms and a 783-bp fragment of the mtDNA control region was analysed for 16 shrews. Phylogenetic analyses of mtDNA sequences revealed reciprocal monophyly for the shrew species, concordant with morphological diagnosis, and supported the idea that the transect cuts through a secondary contact zone between well-differentiated *B. brevicauda* and *B. hylophaga*. In contrast to this pattern, the parasitic nematode mtDNA phylogeny was not subdivided according to host affiliation. Genealogical discordance between parasite and host phylogenies suggests extensive gene flow among parasites across the host species boundary.

Keywords: *Blarina*, contact zone, *Longistriata*, mitochondrial DNA

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Introduction

Most studies of coevolutionary interactions among parasites and hosts have been focused at high taxonomic levels and macrogeographical scales. Typically, alternative scenarios of coevolution, such as host-switching or cospeciation, are tested by gauging the degree of concordance between higher-level phylogenies derived from parasites and their host taxa (e.g. Brooks & McLennan 1993; Hafner *et al*. 1994). Few parasitological studies have documented the population genetic structure of cospeciation (Rannala & Michalakis 2003); therefore, the connection between micro- and macroevolutionary events affecting host–parasite interactions remains poorly explored (but see Mulvey *et al*. 1991; Blouin *et al*. 1992, 1995; Dybdahl & Lively 1996). Patterns of population structure illuminating early stages of differentiation between hosts and parasites can be explored by focusing on areas of contact between two closely related species of hosts.

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The goal of this study is to examine the mtDNA phylogenetic structure of populations of a common parasitic nematode in relation to that of its hosts, the short-tailed shrews *Blarina brevicauda* and *B. hylophaga* (Soricidae: Insectivora). We consider the question of cospeciation between host and parasite in a well-documented and narrow contact zone (0.64–2.94 km) between these species of shrews in central North America (Benedict 1999; Brant & Orti 2002, 2003). The parasitic nematode, *Longistriata caudabullata* (Trichostrongyloidea: Heligmosomidae) lives in the upper small intestine of the shrew and is unique to species of *Blarina* (Dikmans 1946; Vaucher & Durette-Desset 1978; Brant, pers. obs.). Adult worms mate in the intestine and produce eggs that are deposited with the faeces of the shrew. Larval stages of the nematode develop in the faeces and infect the next shrew by ingestion or penetration of juveniles. As in many parasitic nematodes, *L. caudabullata* has negligible dispersal ability and gene flow is thus contingent on the dispersal ability of the host (Blouin *et al*. 1995). Therefore, it seems reasonable to predict that such a parasite — with no intermediate host, no mobile free-living stage and infecting hosts with low vagility — will exhibit a concordant phylogeographical pattern with respect to its hosts

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Table 1 Shrew and nematode samples included in this study

Host specimen*	Nematode individual†	Field number	Collection locality (County, State)	Latitude–longitude (N; W)	GenBank no. for shrews	GenBank no. for nematodes
Blarina brevicauda						
B1	B1.1	1021	Dixon, NE	42.5899; 96.7042	AY121897	AY121866
	B1.2					AY121892
B2	B2.1	1097	Manitoba, Canada	49.1; 95.9833	AY121898	AY121867
	B2.2					AY121887
B3	B3.1	1290	Lancaster, NE	40.6349; 96.5006	AY121900	AY121881
	B3.2					AY121872
B4	B4.1	1291	Lancaster, NE	40.6225; 96.5042	AY121901	AY121878
	B4.2					AY121874
B5	B5.1	1294	Lancaster, NE	40.5911; 96.5016	AY121899	AY121875
B6	B6.1	1295	Lancaster, NE	40.6225; 96.5042	AY121902	AY121880
	B6.2					AY121876
B7	B7.1	1299	Lancaster, NE	40.6067; 96.5016	AY121905	AY121879
Blarina hylophaga						
H1	H1.1	1161	Lancaster, NE	40.5411; 96.4789	AY121893	AY121877
H2	H2.1	1164	Lancaster, NE	40.5689; 96.4783	AY121894	AY121884
	H2.2					AY121869
	H2.3					AY121868
H3	H3.1	1293	Lancaster, NE	40.5667; 96.5019	AY121895	AY121885
	H3.2					AY121888
H4	H4.1	1202	Montgomery, KS	37.012; 95.836	AY121896	AY121865
	H4.2					AY121890
Putative hybrids						
PH1	PH1.1	1165	Lancaster, NE	40.5689; 96.4783	AY121906	AY121891
PH2	PH2.1	1292	Lancaster, NE	40.6102; 96.5002	AY121904	AY121882
PH3	PH3.1	1297	Lancaster, NE	40.5733; 96.5013	Not sequenced	AY121886
	PH3.2					AY121870
	PH3.3					AY121889
PH4	PH4.1	1298	Lancaster, NE	40.5733; 96.5013	Not sequenced	AY121873
PH5	PH5.1	1307	Lancaster, NE	40.5717; 96.5013	AY121903	AY121871
	PH5.2					AY121883

*B = *Blarina brevicauda,* H = *B. hylophaga,* PH = putative hybrid (*B. brevicauda* × *B. hylophaga*) (see Materials and methods for determination of putative hybrids). †*Longistriata caudabullata* isolated from host specimens shown in left column.

(e.g. Blouin *et al*. 1992; Blouin *et al*. 1995; Blouin *et al*. 1999). The main question addressed in this study is whether the interspecific boundary between hosts has an isolating effect on the parasites that may be revealed by mtDNA markers.

Materials and methods

Sample collection and identification

Blarina brevicauda, *B. hylophaga* and their parasitic nematode *Longistriata caudabullata* were collected in a north to south transect of the contact zone (~5.6 km wide) between the two species of shrew in southern Nebraska (Table 1; Benedict 1999). Additional shrews collected well outside the contact zone (from extreme north and southeastern Nebraska, Kansas and Canada; Table 1) were used as a reference for pure parental individuals. Only shrews infected with *L. caudabullata* were used in the analyses (Table 1). In order to verify host specificity, other small mammals syntopic with the shrews were collected and examined for the presence of *L. caudabullata.* Short-tailed shrews were trapped in Sherman live traps set along small mammal runs along roadside ditches with dense vegetation. Shrews were designated to each species or to putative hybrids between species in the field by foot length, as outlined by Benedict (1999).

Laboratory procedures

Total DNA from host tissue was isolated by standard proteinase-K digestion, phenol/chloroform extraction, and purified by ethanol precipitation (Sambrook *et al*. 1989). Total DNA was isolated from the nematodes using the DNeasy Tissue Kit (Qiagen™). Mitochondrial markers were amplified for both the shrews (control region) and the nematodes (NADH dehydrogenase 4 gene, ND4) using the polymerase

chain reaction (PCR). For the shrews, a 780-base pairs (bp) fragment of the control region was sequenced for all samples listed in Table 1. The following primers were used for PCR and sequencing (Stewart & Baker 1994): SDF 5′-CCCCA-CCATCAGCACCCAAAGC-3′ and SDR 5′-AGCGGGTT-GCTGGTTTCACG-3′. For the nematodes, an 800-bp fragment of the ND4 gene was sequenced for all samples listed in Table 1. The following flanking primers for PCR were used: ND4F7: 5′-TTATTTTATGCTAGTCTTTGTTC-3′ and CO3R3: 5′-CTAGTACCCACCATACCAGAT-3′. The following were used as internal sequencing primers: ND4F2: 5′-TGATTT-TTTTATTTCTTGAGAG -3′, ND4F6: 5′-AGTTAGATGT-GTTTTTCA-3′, ND4R1: 5′-CAAAAATAAAGAATAAT-AAA-3′, ND4R2: 5′-GTGTGTTACTGAAGAATAAG-3′, ND4R4: 5′-GCATCACTTTGAAAAACACA-3′. The purified PCR product was sequenced with each primer using the BigDye terminator kit (Applied Biosystems) and determined directly with an ABI 310 analyser (Applied Biosystems). Nucleotide sequence data obtained have been deposited in GenBank (Table 1).

Phylogenetic analysis

Control region and ND4 sequence data were aligned using ClustalW (Gibson *et al*. 1996) with default gap penalties. Phylogenetic analyses were conducted using PAUP* version 4.0b4 (Swofford 2000). Maximum parsimony (MP) analyses were performed with heuristic searches, stepwise additions, replicated 1000 times, with each replicate starting with random input order of sequences with the TBR branch-swapping algorithm. For the shrew data set, bootstrap analysis with 1000 pseudoreplicates was used to measure nodal support; each pseudoreplicate used heuristic searches, TBR and a single starting tree. An optimal model of nucleotide evolution for maximum likelihood (ML) and minimum evolution (ME) analyses was determined by using MODELTEST 3.0 (Posada & Crandall 1998) for both the shrew and nematode data set. The ML and ME trees were obtained by stepwise addition starting trees, TBR swapping algorithm and random addition sequence with one replicate for ML and 100 replicates for ME. Bootstrap analysis used 100 pseudoreplicates, each based on a single heuristic search with a starting tree obtained by random stepwise addition and TBR branch-swapping.

Genealogical concordance

Two methods were used to assess genealogical concordance between the nematodes and the shrews. First, a ‘strict coevolution’ constraint tree was constructed to group mtDNA haplotypes of *L. caudabullata* according to their host affiliation, such that all nematodes parasitizing each of the two species of shrews form monophyletic groups. In order to test the strict coevolution hypothesis, the best tree consistent with this topology was compared with unconstrained optimal trees using the Kishino and Hasegawa test under the ML criterion (Kishino & Hasegawa 1989). The ‘strict coevolution’ scenario constitutes a valid a priori hypothesis that may be tested by this approach (Goldman *et al*. 2000), because it is derived from the previously known host mtDNA phylogeny (Brant & Orti 2002) and the parasitic lifestyle of the nematodes.

Second, the cladogram estimation procedure and nesting rules described by Templeton *et al*. (1992) were used to construct a nested cladogram network. Estimation of the 0.95 probability limit for parsimony and of the network connections were obtained with the program TCS version 1.0 (Clement *et al*. 2000). The geographical structure of the nested genealogy, based on information of geographical origin of the haplotypes, was tested using nested clade analysis (Templeton & Sing 1993).

The nested clade design for nematode haplotypes also was used to test the ‘strict coevolution’ hypothesis. The association between parasite genotype (haplotype) and host (*B. brevicauda* or *B. hylophaga*) was tested with a nested contingency test (Templeton & Sing 1993). Contingency tables of haplotype by host were constructed that recorded the number of nematode haplotypes from each host for each of the nested clades. An exact permutational contingency test using a Monte Carlo technique with 1000 permutations (Roff & Bentzen 1989) was applied to test this association using the program CHIPERM version 1.2 (by D. Posada).

Nucleotide diversity (π) was used to measure DNA polymorphism (average number of nucleotide differences per site between two sequences; Nei & Li 1979). Population parameters were estimated using the DnaSP software version 3.53 (Roza & Roza 1999).

Results

Field collections

Collections produced 13 individuals of *B. brevicauda*, five individuals of *B. hylophaga* and five putative hybrids (infected and uninfected shrews). Among these, the prevalence and range of intensity of the parasite *L. caudabullata* in the contact zone in *B. brevicauda* were 38% and 1–30 individuals per host, respectively. For *B. hylophaga* these values were 60% and 2–20 individuals, and among the five putative hybrids prevalence was 100%, with 5–15 individuals per host. No individuals of *L. caudabullata* were found in the other small mammals collected with the shrews.

General pattern of sequence variation

Sixteen of the 23 shrews collected were parasitized and control region sequenced. However, two of the 16 could

not be sequenced successfully (PH3 and PH4; Table 1). A total of 783 bp, about 75% of the control region, was analysed for 14 individuals of *Blarina* (Table 1). This data set contained 515 constant sites, 110 variable and parsimony uninformative sites and 158 parsimony informative sites. This variation defined 14 different haplotypes, i.e. no identical haplotypes were found among the 14 individual shrews sequenced. The HKY + G model (Hasegawa *et al*. 1985; Yang 1993) was selected by MODELTEST (Posada & Crandall 1998) as the best fit for the control region sequences. Parameters estimated for this model were: Ti/Tv = 2.1035, gamma shape parameter = 1.05, base frequencies A = 0.3693, C = 0.2412, G = 0.0835, T = 0.3031. Maximum percentage sequence difference (uncorrected) among *B. hylophaga* haplotypes was 2.7%, minimum distance was 0.6% and the average was 1.8%. For *B. brevicauda* these values were: maximum = 13%, minimum = 0.3%, and average = 5.9%. Average sequence distance between *B. brevicauda* and *B. hylophaga* was 9.4%.

Between one and three individual *L. caudabullata* nematodes were sequenced from each of the parasitized shrews (total = 28, Table 1). A fragment of 800 bp, about 75% of the ND4 gene plus about 92 bp of the intergenic region flanking the ND4 gene was sequenced. The complete data set contained 730 constant sites, 46 variable uninformative sites and 24 variable informative sites. This variation defined 27 distinct haplotypes, with two individuals sharing the same haplotype. The HKY + I + G model (Hasegawa *et al*. 1985; Yang 1993; Gu *et al*. 1995) was selected by MODELTEST as the best fit for the ND4 data set (Posada & Crandall 1998). Parameters estimated for this model were: Ti/Tv = 2.633, Pinvar = 0.7582, gamma = 0.6519, base frequencies A = 0.2733, C = 0.1006, G = 0.1628, T = 0.4638. Maximum percentage sequence difference among haplotypes was 3% (B7.1 vs. B4.1 and PH3.3 vs. B4.1) and minimum was 0.12%. For nested clade analysis, we excluded the highly variable intergenic region. Inclusion of this region resulted in nonparsimonious connections in the network estimation procedure. Excluding the intergenic region reduced the number of haplotypes from 27 to 25.

Phylogenetic inference

The topology of the ME tree was identical to that of the ML tree for the shrews. The monophyly of both species was well supported by all analyses. Maximum parsimony analysis of the shrew control region resulted in four equally parsimonious trees. In agreement with our previous study (Brant & Ortí 2002), the MP and ML host haplotype trees show two well-supported groups corresponding to individuals identified as *B. brevicauda* and *B. hylophaga* (trees not shown, see bootstrap values on ML tree, Fig. 1a).

Maximum parsimony analysis of the nematode ND4 region resulted in over 10 000 MP trees (length 105, CI = 0.771, RI = 0.692). The consensus tree supported only grouping of (B4.2, H1.1, B7.1) and (B3.2, PH3.3, PH1.1) found by ML analysis (Fig. 1b).

Tests of genealogical concordance

Segregation of nematodes by host species was not supported by phylogenetic analysis (see ML results in Fig. 1b). Enforcing the 'strict coevolution' constraint tree on the original data set resulted in four ML trees with –log L = 1746.4564 (unconstrained –log L = 1684.2327). The likelihood ratio test rejected the constrained trees ($P = 0.002$) in favour of the best tree (Fig. 1b) that shows no genetic break among the nematodes affiliated to different host species.

The TCS network with most parsimonious connections for the ND4 haplotypes of *L. caudabullata* had four alternative topologies resulting from an ambiguity loop. Each of the four topologies was evaluated by breaking one of the alternative connections and nesting again the diagram, then running the permutation and GeoDis b2.0 (Posada *et al*. 2000) tests on each one. The null hypothesis of no association between nematode haplotype distribution and geographical location could not be rejected in any case. The same result was obtained for the tests of host association. Due to sample size, contingency tables were constructed only for higher-level clades (three-step and four-step clades). All nested contingency permutation tests failed to reject the null hypothesis of no association between nematode haplotype and host (all P-values were > 0.5). Transmission of parasitic nematodes and therefore gene flow among their subpopulations does not appear to be restricted to closely related hosts.

Discussion

This study illustrates how an empirical question of host–parasite cospeciation can be analysed effectively at the microevolutionary level. The low vagility of the host, the absence of known intermediate hosts and of mobile stages in the parasite life cycle lead us to predict cospeciation of shrews and nematodes, resulting in population subdivision and concordant mtDNA genealogies. However, the discordant phylogeographical pattern of nematodes and shrews indicates that the interspecific boundary between hosts is 'invisible' for the parasites, or at least that the speciation event separating *B. hylophaga* and *B. brevicauda* is not reflected in the distribution of mitochondrial genes in the parasites. The speciation event separating *B. brevicauda* and *B. hylophaga* was estimated to about 4.6 ma (Brant & Ortí 2002). It is interesting that after such a long period of isolation no evidence — molecular or morphological — of even incipient stages of speciation can be detected among parasites isolated from the different species of shrews. Although our sample size is somewhat limited, the results are unambiguous.

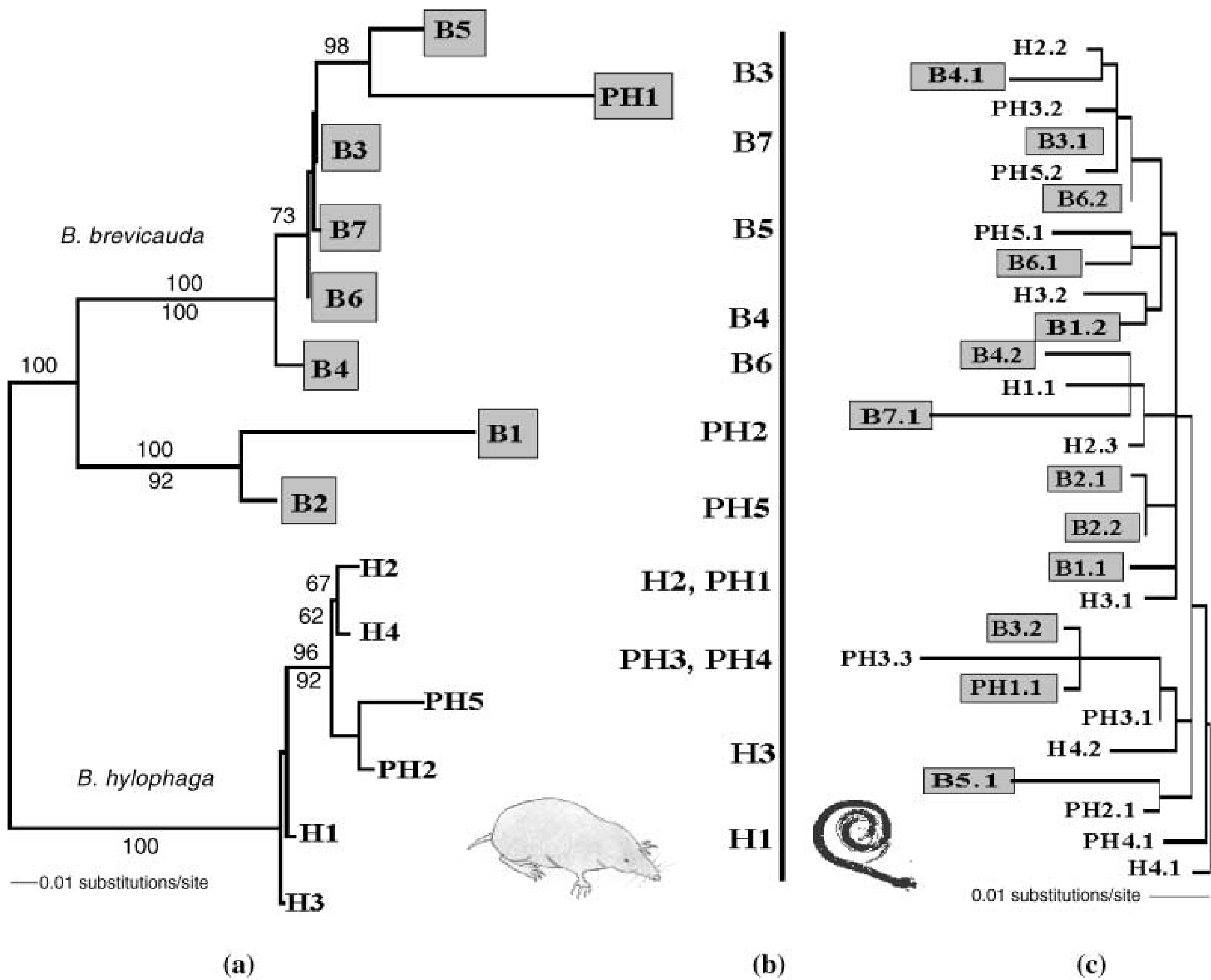

Fig. 1 (a) Maximum likeihood tree for *Blarina brevicauda* and *B. hylophaga* mitochondrial control region sequences based on the HKY + G model (see text for parameters). Bootstrap values (50% majority rule) for ML are shown above the branches, MP bootstrap values below the branches. Letters and numbers follow the species and locality, respectively, outlined in Table 1. (b) The solid black line with haplotype designations represents the collection localities in southeastern Nebraska within the area of contact between the two species of shrews (~5.6 km). Shrews B1, B2 and H4 were collected from the reference population outside the contact zone. (c) Maximum likelihood tree for individuals of *Longistriata caudabullata* mitochondrial ND4 sequences based on the HKY + G + 1 model (see text for parameters). Bootstrap values (50% majority rule) for ML are shown above the branches. Letters and numbers follow the species and locality, respectively, following Table 1. Shaded individuals are nematodes colected from *B. brevicauda*. Although the hosts PH3 and PH4 were not included in the shrew analyses, nematodes from these shrews were included. It should be noted that the mitochondrial region (noncoding region) evolves much faster than the ND4 protein (protein coding); therefore, branch lengths are not equivalent for a direct comparison.

Several possible scenarios may explain the observed pattern of discordance. These include host-switching, contemporary gene flow masking historical fragmentation, incomplete lineage sorting, variable molecular evolution between the interacting species, dissimilar effective population sizes, differential selection pressures on the interacting species and/or ecological differences (life cycles) (e.g. Mulvey *et al*. 1991; Brooks & McLennan 1993; Dybdahl & Lively 1996; Althoff & Thompson 1999). The most relevant possibilities for this system are discussed below.

Compared to the shrew mtDNA phylogeny, the nematode tree is shallow and largely unresolved (Fig. 1). The major difference in absolute genetic divergence values between mtDNA haplotypes observed within *L. caudabullata* and *Blarina* species may be explained by significant differences in the relative rates of nucleotide substitution between the control region (shrews) and the ND4 gene (nematodes). Although no direct comparison has been made across these taxa, the noncoding mitochondrial control region evolves at least four times faster than mitochondrial protein-coding genes among vertebrates (e.g. Avise 2000 and

references therein). However, in spite of this predictably lower sequence divergence among ND4 sequences, lack of resolution in the phylogenetic tree (Fig. 1) is not necessarily implied. Low sequence divergence and a 'star phylogeny' also would be consistent with a fast-growing population expanding after a recent colonization event. In this view, the nematodes could be seen as a recent acquisition in *B. hylophaga* (following a host-switching event from *B. brevicauda*). However, additional population and demographic factors must be evaluated (Blouin *et al*. 1992; Rannala & Michalakis 2003).

Parasite populations will not necessarily differentiate after a speciation event separates their hosts if the newly formed host species remain in geographical proximity or come into secondary contact, allowing parasite transmission across the species boundary (Brooks & McLennan 1993). In fact, a 'star phylogeny' in *Longistriata* also could have resulted from secondary contact between *B. brevicauda* and *B. hylophaga*. As *B. brevicauda* and *B. hylophaga* diverged, this scenario might assume the isolation of *L. caudabullata* in one but not both incipient species of shrews, or failure of the parasite to speciate. The parasite might have 'missed the boat' (Brooks & McLennan 1993) or have gone extinct in one of the newly formed sister species of host. Then, subsequent to secondary contact, nematodes from one species were able to invade the second species successfully, and undergo a range expansion event.

Because we used sequence data from the ND4 region, comparisons of the genetic variability observed with results from published nematode studies using the same mtDNA region are possible (Blouin *et al*. 1992; Blouin *et al*. 1995; Hoberg *et al*. 1999; Hawdon *et al*. 2001). The haplotype diversity value observed for *L. caudabullata* (0.99) is similar to what has been found in other population samples of trichostrongylid nematodes; however, the nucleotide diversity ($\Pi = 0.012–0.028$ in other trichostrongylid nematodes; Blouin *et al*. 1999) is typically twofold larger, on average, than what we obtained ($\Pi = 0.012$). The values of nucleotide diversity found in this study are more similar to those found in *Necator americanus* (Hawdon *et al*. 2001). If haplotypes and nucleotide diversity can be considered a function of effective population size (Blouin *et al*. 1999), then it is plausible that *L. caudabullata* has a modestly high effective population size. Furthermore, genetic differences between nematodes within a single host (range 0.3–2.3%) compared to all individuals (0.12–3%) were similar, indicating high levels of genetic variation within hosts and between hosts. More individuals would need to be collected to calculate and verify these observed patterns.

In conclusion, the regional pattern of mtDNA uncovered in this study suggests *L. caudabullata* is not genetically specific to a single host species and in fact that it may have recently invaded *B. hylophaga*. It is unlikely that a simple explanation will uncover the mechanism(s) that created the patterns found in this paper. By extending this study with more individuals of nematodes from the other species of *Blarina* and a wider geographical range, plus incorporating lifecycle details, and comparing them to other such works (i.e. Blouin *et al*. 1999; Hoberg *et al*. 1999; Hawdon *et al*. 2001) we will be able to reconstruct the evolutionary history of this host–parasite relationship in light of the patterns uncovered in this work. Clearly, this is an exciting step ahead to narrowing the gap between micro- and macroevolutionary studies of symbiotic organisms. This study will contribute to our overall quest to understand the historical and contemporary patterns and processes characteristic in the coevolution of a host–parasite assemblage.

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This research comprises part of Sara V. Brant's PhD thesis, which was concerned with examining the micro- and macroevolutionary patterns and processes affecting the genetic structure of short-tailed shrews and their nematode parasites. SB is currently a postdoctoral fellow at Louisiana State University examining coevolutionary relationships among gophers and their lice. Guillermo Ortí was the adviser for Sara V. Brant's PhD thesis. His research focuses on using molecular markers to reconstruct the gene genealogies ranging from paternity analysis to deep phylogenies, focusing mainly on the molecular systematics of fishes. For more information, see <http://golab.unl.edu/>.