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Abstract

The parasitic nasal mite *Ptilonyssus sairae* Castro has been classified as one species, but we find that significant sequence divergence exists between host-specific mite populations. The low level of gene flow between conspecifics is probably a result of the parasites' low rate of dispersal among different bird host species. The internal transcribed spacer (ITS) of the nuclear ribosomal RNA gene from *P. sairae* populations was sequenced and used for phylogenetic inference and subsequently compared to their passerine bird host phylogeny. In addition to finding restricted gene flow between host-associated parasites, the reconciliation of host and parasite gene trees reveals statistically significant levels of strict cospeciation between bird hosts and host-specific mite populations. Our findings suggest that cryptic speciation may be common in this group of mites.

Key words: Acari, phylogeny, species delineation, cryptic speciation, coevolution, cophylogeny

Introduction

Explaining differences in host specificity remains a fundamental question in studies of host-parasite coevolution (Thompson 1994). Evolutionary and population genetic studies of host-parasite interactions can provide important information regarding coevolutionary factors, such as host specificity, parasite dispersal, gene flow, and host-associated cospeciation (Johnson *et al*. 2002a). Host-parasite interactions are extremely interesting because they potentially represent a long history of an intimate association between very distantly related taxa. Factors of host specificity in parasites can be a result of several phenomena, and the isolation of such factors provides crucial information into the co-relatedness of host and parasites (Hafner *et al*. 2002).

Though many arthropod host-parasite interactions have been documented, few have been studied in a cophylogenetic framework that seeks to show cospeciation. A textbook example of cospeciation has been documented between pocket gophers and their parasitic chewing lice (Hafner *et al*. 1994). Studies of this system have revealed the potential coevolutionary information that can arise from such work, such as host-parasite ecology, the testing of evolutionary models, and cospeciation (Hafner *et al*. 2002). This study also demonstrates the power of using molecular markers as tools for elucidating ecological relationships between host and parasite.

Other examples of arthropod host-parasite systems studied in a cophylogenetic framework involve cases between bird and lice parasites and are described and summarized by Clayton *et al*. (2004). In this review, cospeciation found between Apodidae and their parasite genus *Dennyus*, as well as between Columbiformes and their parasite genus *Columbicola*, was further substantiated using ecological data and cophylogenetic comparison of lice and hosts. For example, ecological factors examined in governing dispersal capability of *Columbicola* (feather lice) that parasitize pigeons and doves include: dispersal on detached feathers, shared dust baths, shared nest holes, and

phoresy on hippoboscid flies. Here, the relationship between ecology and phylogenetic congruence showed how ecological factors can have a fundamental impact on the "probability of cospeciation, host switching, and other macroevolutionary events that govern the congruence of interacting clades" (Clayton *et al.* 2004).

Passerine birds and their parasitic nasal mites represent an example of how cophylogenetic inferences can shed light onto ecological factors that may govern coadaptive responses between host and parasite. Parasitic nasal mites in the Rhinonyssidae family are obligatory endoparasites that are found in the nares and turbinates of non-ratite birds. These parasites are blood feeders that require the moist and warm microhabitat of the host bird's nasal passage. They can remain in the nasal cavity by using their ambulacral claws or suckers (Mitchell 1963), but the nasal mucus that they are embedded in also probably contributes (Fain 1969).

Mode of transmission among these mites is currently unknown. This is problematic because it makes it difficult to explain host specificity when the means of transfer from one host to another is not fully understood. For example, Porter and Strandtmann (1952) found that House Sparrow nestlings were infected with *Ptilonyssus nudus* Berlese & Trouessart and *P. hirsti* (Castro & Periera) at about a 40% infection rate, while their adult parents were infected at a rate of about 70%. Murray (1966) also noted that young Gouldian Finches were infected with *Sternostoma tracheacolum* Lawrence nasal mites. However, others have noted a different pattern of infection. TerBush (1963) showed that in gulls, which could be aged by plumage patterns, first year birds were only infected with *Larinyssus orbicularis* Strandtmann at about 1%, while second year birds were at 40%, and adult birds showed a rate of about 55%. The extensive laboratory studies of Bell (1996) suggest that transmission of the mite *S. tracheacolum* occurs by adult non-gravid females that migrate out of the nares onto the head plumage, and eventually into a new host.

Prior to Pence and Casto's (1976) extensive study on the variation and morphology of *P. sairae* "complex", 12 distinct species had been attributed to what is now considered one species. Pence and Casto (1976) subsequently concluded that seven species (including *P. sairae*) were actually indistinguishable based on the overlapping and unstable meristic characters that were originally used to differentiate these species. They concluded that there were no discrete characters that could serve to differentiate specimens from different host families. Therefore, *P. japuibensis* Castro, *P. japuibensis cyanocompsae* Fain & Aitken, *P. agelaii* Fain, *P. teretistris* Cerný, *P. ludovicianus* Cerný, and *P. garridoi* Cruz were classified as synonyms and subsequently grouped into one species, *P. sairae*. Since that time, most workers (Spicer 1977a, b, 1978, 1984; Wilson 1980) have agreed with their conclusions.

Key questions regarding host specificity of *P. sairae* remain unanswered. For example, do populations of *P. sairae* that parasitize different hosts represent a complex of closely related species with high host specificity? Or does *P. sairae* represent a single, highly variable species with low host specificity? Since characters previously used in differentiating species of the *P. sairae* complex are subject to significant variability, such questions can be more accurately addressed using molecular data. Molecular analyses allow us to study host-parasite systems with regards to the scope of host specificity. Molecular data allows for a more confident evaluation and inference of host and parasite phylogenies, in addition to detecting population level differences that would otherwise not be possible using morphological characters. Often morphological information provides few homologous characters to use in phylogenetic studies, as selection pressures cause a convergence in morphology (Downes 1990). Consequently, morphological data for phylogenetic studies may contain high levels of homoplasy (Dabert J. *et al.* 2001), in which character states resembling each other are not the result of common ancestry.

Studies inferring molecular phylogenies of rhinonyssid nasal mites have used the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) genes and the mitochondrial

16S rRNA gene (de Rojas *et al*. 2001, 2002; Ubeda *et al*. 2003). As an example, de Rojas *et al*. (2002) used the ITS region which showed levels of variation at 1–8% divergence among morphologically different species in the genus *Sternostoma*, and 13–44% divergence was found among different morphological species in the genus *Tinaminyssus*. A number of studies have shown ITS to be useful for genetically differentiating between closely related mite taxa, given that the ITS region evolves rapidly and allows for between-species variation (Navajas 1998; Essig *et al*. 1999; Navajas *et al*. 1999; Ramey *et al*. 2000; Yli-Mattila *et al*. 2000; de Rojas *et al*. 2002; Dabert M. *et al*. 2005). Navajas and Fenton (2000) and Cruickshank (2002) provide a thorough discussion of the utility of the different genes employed in mite phylogenetic studies.

In this study, we have three objectives: the first to determine *P. sairae* "intraspecific" genetic variation; the second to determine if the genetic differentiation shown is host-specific; and the third to test for evidence of cospeciation. To address these questions, we sequenced the ITS region of the nuclear rDNA gene region (ITS1, 5.8S rDNA, ITS2) of *P. sairae* individuals from the passerine 'Nine-primaried Oscine' bird host families Emberizidae and Parulidae. Lastly, although there has been molecular work done on nasal mites, none of these studies have looked at cospeciation. Therefore, we compared the molecular phylogeny of *P. sairae* to their passerine bird host phylogeny to investigate evidence of cospeciation.

Materials and methods

Specimen collection. Bird specimens were collected during the summers of 2000, 2001, and 2003 and immediately placed on wet ice in the field and subsequently transferred to a –80ºC freezer. Nares and turbinates of birds were examined under a dissecting microscope for the presence of nasal mites. Birds were dissected and the mites removed with a probe to ensure that all mites in the bird were collected. When mites were collected, one was mounted as a voucher specimen, and the others were put in 10X TBE buffer and stored at –80°C for subsequent DNA extraction.

DNA extraction. One single mite was used for each DNA extraction procedure, which permitted the population level nature of this study. Each mite was frozen with liquid nitrogen and then crushed with a pestle to destroy its tough, chitinous exoskeleton. Thirty µl of lysis buffer (Williams *et al*. 1992) was immediately added to the tube with the crushed mite, and then DNA was extracted from mites using the protocol from Williams *et al*. (1992) with some modifications. A drop of mineral oil was added to the mixture in the tube, and then subsequently subjected to a three step temperature cycle: 60ºC for 70 minutes; 95ºC for 15 minutes (to deactivate the Proteinase K); and a final cool down at 14ºC.

PCR amplification. The complete internal transcribed spacer 1 (ITS1), 5.8s rDNA, and ITS2 region of the ribosomal RNA gene was amplified using mite specific primers from Navajas *et al*. (1998) that have been used for other rhinonyssid mites by de Rojas *et al*. (2002). Mite specific primers are as follows: forward 5'-AGAGGAAGTAAAAGTCGTAACAAG-3' and reverse 5'-ATATGCTTAAATTCAGGGGG-3'. An additional mite-specific amplification and sequencing primer was developed to obtain cleaner sequences and also to obtain sequence from Lark Sparrow (*Chondestes grammacus*) parasites. The sequence is as follows: 5'-GGTCTTCACATYTGATTTCAG 3'. Double stranded PCR were run in 26 µl reactions consisting of 2 µl DNA template, 2 µl 100% DMSO, 5 µl 10 mM dNTP, 5 µl of each 10 µM primer, 10 µl 5X buffer D (Invitrogen: 300 mM Tris–HCl, 75 mM $(NH_4)SO_4$, *p*H 8.5, and 17.5 mM $MgCl_2$), 2 µl distilled water, and 0.2 (units) µl of 10X Amplitaq Polymerase (Applied Biosystems, Foster City, CA). Reaction parameters include: 94ºC initial 2-minutes denaturation; 35 cycles of 94ºC for 30s, 54ºC for 30s, and 72ºC 2 mins; 72ºC final extension; and finally, a 14ºC soaking.

DNA sequencing. Prior to cycle sequencing, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The region was sequenced in both directions. Cycle sequencing reactions were done in 12 μl reactions: 1 μl ABI Prism BigDye v 3.1 (Applied Biosystems, Inc. 1998), 1.5 μl 5X buffer (400mM Tris at *p*H 9.0 and 10 mM MgCl$_2$), 0.5 μl 100% DMSO, 0.5 μl of 10 μM primer. The remainder of the cocktail consisted of PCR water and DNA template to give 50–90 ng of template DNA in each reaction. Cycle sequence reaction parameters were 25 cycles of 96ºC for 30s, 60ºC for 30s, and 60ºC for 4 minutes. Cycle sequencing products were cleaned using the isopropanol precipitation method (Applied Biosystems, Inc. 1998). Reaction products were resuspended in 2.5 μl of deionized formamide (25 mM EDTA with 50 mg/ml Blue Dextran) solution. Resuspended samples were vortexed, denatured at 90ºC for 2 minutes and immediately placed on ice until loading onto an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) following manufacturer's protocol.

Sequence Alignment and Phylogenetic analysis. Sequences were aligned using the computer programs Sequencing Analysis 3.4 (Applied Biosystems, Inc. 1999) and Sequencher 3.1.1 (Gene Codes Corporation 1998). Base composition bias was calculated according to Irwin *et al*. (1991). To infer phylogenetic relationships, both parsimony and maximum likelihood (ML) analyses were performed using PAUP 4.0b10 (Swofford 2001). Parsimony analysis was executed using the branch-and-bound algorithm that guarantees finding the shortest tree. All characters were unordered and equally weighted. A bootstrap analysis using a heuristic search of 800 closest stepwise addition replicates provided support for parsimony branching patterns (Felsenstein 1985). Maximum likelihood models were determined using Modeltest version 3.7 (Posada and Crandall 1998). A Jukes-Cantor neighbor-joining tree with its estimated parameters based on the best-fit model was used to begin an iterative maximum likelihood analysis. This was accomplished by using a neighbor-joining as a starting tree option and conducting an iterative heuristic search until the tree parameters converged (Sullivan *et al*. 2005). To evaluate support for the maximum likelihood tree branches, a bootstrap analysis using a heuristic search of 800 random stepwise addition replicates was conducted (Felsenstein 1985).

Host-parasite tree reconciliation. A tree reconciliation analysis was performed with the *P. sairae* mite parasite phylogeny and corresponding bird host phylogeny (Page 1990, 1994). The bird host phylogeny was constructed from a subset of Carson and Spicer's (2003) extensive phylogenetic analysis, in which 38 species of North American Emberizid sparrows based on 3 mitochondrial genes (1673 base pairs) were analyzed. We took bird hosts from which nasal mites were extracted, and only used those parasitized taxa to produce our host phylogeny. Tanglegram of the nasal mite parasite *P. sairae* and its 'Nine-primaried Oscine' bird hosts (Emberizidae and Parulidae) were implemented and analyzed in TreeMap (Page 1995). *Ptilonyssus pirangae* (Cerný) was used as the outgroup.

Results

Specimens sequenced, hosts in which parasites were found, localities, and Genbank Accesion numbers are listed in Table 1.

The final aligned ITS sequence is 473 base pairs. Of the 473 base pairs, 143 sites are variable, and 139 sites are parsimony-informative. Mean base composition with all characters included is as follows: A = 0.324; C = 0.224; G = 0.148; T = 0.304. Mean base composition of variable characters is as follows: A = 0.281; C = 0.228; G = 0.166; T = 0.325. Base composition bias for all characters is 0.171, with the base composition bias of variable characters of 0.141. The Chi-square test for homogeneity of base frequencies across sequences revealed no heterogeneity in each case.

TABLE 1. Number of individual mite specimens sequenced, hosts in which parasites were found, localities, and GenBank accession numbers for *Ptilonyssus sairae*. The mite from *Piranga ludoviciana* was used as the outgroup and is a separate species, *P. pirangae*.

Specimens Sequenced	Bird host	Locality	GenBank accession	Host family
A - 1 sequenced	*Spizella passerina* Chipping Sparrow	Sierra, Sierra County, USA	EU190986	Emberizidae
B- 3 sequenced	*Spizella passerina* Chipping Sparrow	Hopland, Mendocino County, USA	EU190987	Emberizidae
C - 1 sequenced	*Chondestes grammacus* Lark Sparrow	Hopland, Mendocino County, USA	EU190988	Emberizidae
D - 2 sequenced	*Junco hyemalis* Dark-eyed Junco	San Bernadino County, USA	EU190989	Emberizidae
E - 2 sequenced	*Junco hyemalis* Dark-eyed Junco	Mt. Pinos, Ventura County, USA	EU190990	Emberizidae
F - 1 sequenced	*Junco hyemalis* Dark-eyed Junco	Los Padres, Monterey County, USA	EU190991	Emberizidae
G - 1 sequenced	*Pipilo chlorurus* (Aud.) Green-tailed Towhee	Mt. Pinos, Ventura County, USA	EU190992	Emberizidae
H - 2 sequenced	*Dendroica coronata* Yellow-rumped Warbler	Sierra, Sierra County, USA	EU190993	Parulidae
I - 3 sequenced	*Dendroica coronata* Yellow-rumped Warbler	Sierra, Sierra County, USA	EU190994	Parulidae
J - 1 sequenced	*Piranga ludoviciana* Western Tanager	Sierra, Sierra County, USA	EU190995	Thraupidae

Uncorrected pairwise genetic divergences (p-distance) are listed in Table 2. The *Ptilonyssus sairae* nasal mites that were found parasitizing the same host individual in all host species yielded no genetic difference. These intra-host genetic divergence comparisons included the following: D-1 and D-2 (host: *Junco hyemalis* (Linnaeus)); E-2 and E-3 (host: *J. hyemalis*); H-1 and H-2 (host: *Dendroica coronata* (Linnaeus)); I-1, I-2, and I-3 (host: *D. coronata*). Mites that were found on the same host species, but different individual host specimens, yielded little to no genetic divergence (0% to 0.2% intra host-species genetic divergence). This is shown for mites parasitizing two different host individuals of *Spizella passerina* (Bechstein), three different host individuals of *J. hyemalis*, and two different host individuals of *D. coronata*. Of these three intraspecific comparisons, only one mite from the *Junco* comparison showed any divergence. Mites that were found on different host species yielded genetic divergences that ranged from 5.9% (*S. passerina* vs *Chondestes grammacus* (Say)) to 17.6% (*D. coronata* vs *J. hyemalis*). Sequence divergence ranged from 20.2% to 21.0% for the mite *P. pirangae* found on *Piranga ludoviciana* (Wilson) bird host, which was used as the outgroup.

Four most parsimonious trees were found with a length of 173. For the maximum likelihood analysis, Modeltest (Posada & Crandall 1998) suggested the same model from both the LRT and the AIC best-fit models tests for these data, which was HKY85+G. The iterative ML search utilizing this model, resulted in one maximum likelihood tree (Figure 1) with a -lnL = 1401.11568. This was the same as the strict consensus of the four parsimonious trees and was considered to be the best estimate of the phylogeny. The parameter values estimated from this tree were ti/tv ratio of 1.6868, α = 0.34820 for the Γ distribution, and estimated base composition as A = 0.3214, C = 0.2190, G = 0.1518, T = 0.3076.

TABLE 2. Uncorrected pairwise percent genetic divergences of host-specific populations of the nasal mite *Ptilonyssus sairae.* Descriptions in parentheses indicate individual host specimen (letter) and individual mite specimen (number) represented in Table 1 and Figure 1.

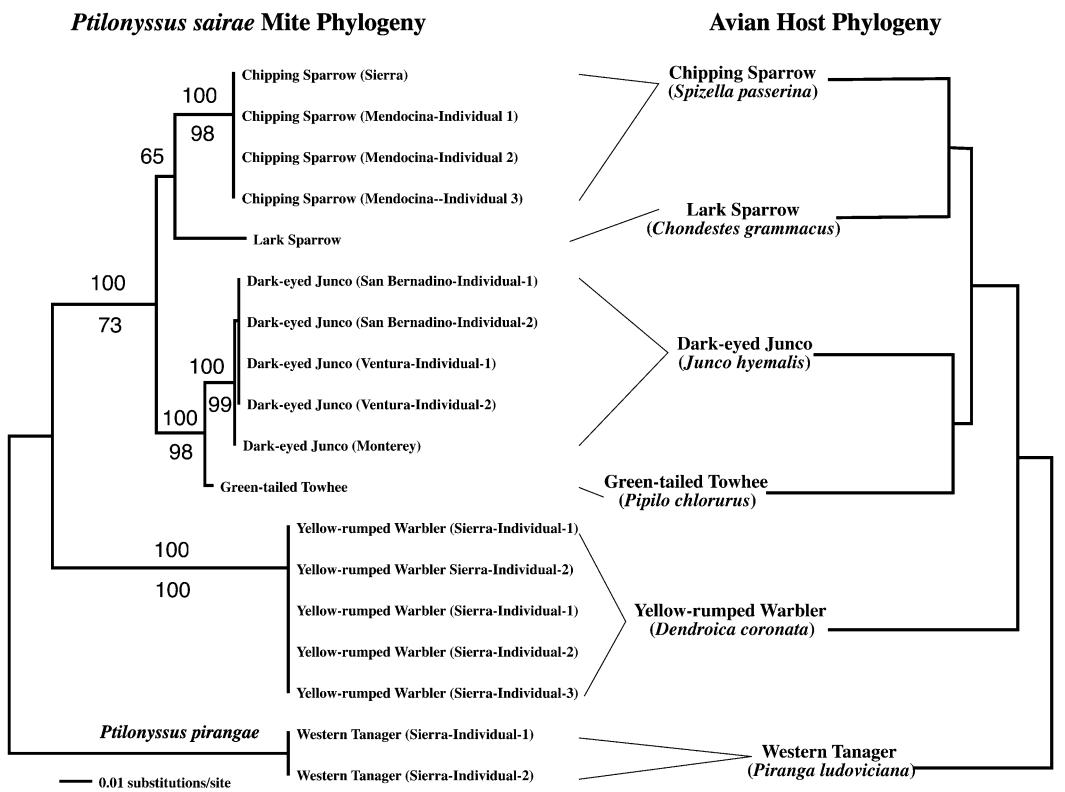|	Chipping Sparrow (A-1)	Lark Sparrow (C-1)	Dark-eyed Junco (D-1)	Green-tailed Towhee (G-1)	Yellow-rumped Warbler (H-1)
Chipping Sparrow (A-1)	-				
Lark Sparrow (C-1)	5.99	-			
Dark-eyed Junco (D-1)	7.33	8.00	-		
Green-tailed Towhee (G-1)	6.11	6.58	2.03	-	
Yellow-rumped Warbler (H-1)	17.28	16.37	17.59	16.89	-
Western Tanager (J-1)	20.44	21.97	21.34	20.20	21.11

FIGURE 1. Tanglegram of the nasal mite parasite *Ptilonyssus sairae* and its 'Nine-primaried Oscine' bird hosts showing strict cospeciation. The mite phylogeny is the Maximum Likelihood (HKY85+G) phylogeny (-lnL=1401.11568), and the strict consensus of the four most parsimonious trees (L=173) as inferred from ITS sequence. The boostrap values above a line indicate parsimony support, while the values below a line indicate ML support. The bird phylogeny is based on three mitochondrial genes and is taken from Carson and Spicer (2003). Five cospeciation events are present, and this represents statistically significant cospeciation (P=0.0095).

A tanglegram of the nasal mite parasite *P. sairae* with bootstrap values and its bird host phylogeny as implemented in TreeMap (Page 1995) is shown in Figure 1. Five strict cospeciation events are shown, representing a statistically significant level of cospeciation (P=0.0095).

Phylogenetic analysis of host-specific populations of *P. sairae* displayed significant sequence divergence and branching patterns consistent with its corresponding bird host branching patterns.

Discussion

The question of delineating species boundaries in molecular evolutionary genetics remains a fundamental concern, because there does not exist an objective cutoff in which one could indicate that no significant gene flow is occurring between populations, and thus deserves species status (Wiens & Servedio 2000). However, it has been noted by Dobler and Farrell (1999) that for parasitic organisms "gene flow between parasite populations on different host species can be limited even if the parasites are not 100% specific. Such structure may favor formation of host races, or speciation". The species problem in general and its designation using molecular data has been debated once again in the current literature (Mallet 1995, Agosti 2003, Baker *et al*. 2003, Lipscomb *et al*. 2003, Mallet & Willmott 2003, Seberg *et al*. 2003, Tautz *et al*. 2003). There currently exist several methods by which delineating species can be attempted, not to mention the multitude of species concepts and definitions in the literature. Sites and Marshall (2004) provide a concise review of a handful of these methods, as well as the associated types of data and assumptions. Of the 12 empirical methods of species delineation that Sites and Marshall (2004) describe, one particular empirical method of species delineation seems to fit our data set best (the "M/GC" method). This method equates the reduction or absence of gene flow as species boundaries, with the assumption that the between-species variability is greater than the within-species variability. The genetic variation of *P. sairae* parasites inhabiting the same host species displayed little or no genetic differentiation, although genetic variation proved to be significant in parasites inhabiting different host species. From this we can conclude that host-specific populations of nasal mites do not appear to be exchanging genetic material. If then, we call host-specific populations of *P. sairae* "species", then we can in fact delineate species boundaries based on the evidence of the absence of gene flow. In addition, the genetic differentiation found between host-specific populations of *P. sairae* is typical of the between-species genetic divergence found between nasal mite parasites that are morphologically distinct (Navajas *et al*. 1999; de Rojas *et al*. 2002), which for the ITS region is typically between 5–30%. However, we are currently not suggesting a taxonomic revision of *P. sairae* into separate host-specific species, but we do present suggestive evidence that host-specific populations of parasites are following different evolutionary trajectories due to the dependence of parasite dispersal on its ecology and host behavior.

Do populations of *P. sairae* that parasitize different hosts represent a complex of closely related species with high host specificity, or does *P. sairae* represent a single, highly variable species with low host specificity? Further understanding of gene flow in populations of parasites is essential to effectively interpret similarities in host and parasite phylogenies. In this instance, questions of bird-mite host specificity still require further investigation. Our data set presents obvious limitations with regards to equating DNA haplotypes to species, though we demonstrate evidence to support host-specific driven speciation. However, this data set involves only one locus in the nuclear genome, and there is not an objective criterion for determining sufficient number of unlinked loci required to delineate coalescent species (Hudson & Coyne 2002). Additionally, another important consideration is that methods that rely on gene genealogies can be misleading if divergence has been recent (Avise 2000), and in this instance it has been recent. However, if additional studies confirm our finding of cryptic speciation in rhinonyssid nasal mites, then it has interesting implications for the number of species in the family Rhinonyssidae. From the taxonomic perspective, probably only about half the number of morphological species (~500) in the family Rhinonyssidae have been described, given

that so few tropical bird species have been examined for nasal mites. But this may be an extreme underestimate of the true number of evolutionary species if our preliminary molecular studies are an accurate indication. They suggest that each bird species may have its own species of nasal mite, and since there are approximately 9,000 species of birds, the number of rhinonyssid species might be on the order of 5,000–10,000 species.

Another interesting aspect to our results concerns the observation of strict cospeciation. Assuming that the *P. sairae* host-specific mite populations can be considered as different species, then we found perfect topological congruence between the mite phylogeny and that of the parasitized bird hosts. Although finding significant cophylogeny between bird hosts and arthropod parasites is not unusual (Page *et al*. 1998; Mironov & Dabert 1999; Paterson *et al*. 2000; Dabert J. *et al*. 2001; Johnson *et al*. 2002b; Johnson & Clayton 2003), the discovery of strict cospeciation is not the typical outcome (Johnson & Clayton 2003). It has been recognized that this requires a unique set of conditions between host and parasite (Clayton *et al*. 2004), which may occur in this group of nasal mites. However, as already mentioned, very little is known of the ecology and transmission of nasal mites from host to host, so at the moment it is difficult to assess the factors that might contribute to strict cospeciation if it is indeed occurring. Additionally, it is possible that our observation is spurious and just due to our limited sampling, since many more species of birds in the 'Nine-primaried Oscine' group are known to have *P. sairae* nasal mites (Pence & Casto 1976, Spicer 1977a, 1977b, 1978, 1984). Consequently, many more mites from different host species will need to be examined before strict cospeciation in this group can be considered a general observation.

Acknowledgements

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