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HOST SPECIALIZATION DIFFERENTIATES CRYPTIC SPECIES OF FEATHER-FEEDING LICE

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Parasite species with differentiated host-specific populations provide a natural opportunity to explore factors involved in parasite diversification. *Columbicola macrourae* is a species of ectoparasitic feather louse currently recognized from 15 species of New World pigeons and doves. Mitochondrial sequences reveal five divergent haplotype clusters within *C. macrourae*, suggesting cryptic species. Each cluster is relatively host specific, with only one or a few hosts. We conducted a reciprocal transfer experiment with two of these lineages to test whether host use has an adaptive component. Our results demonstrate that the fitness of each lineage is considerably higher on its native host than on the novel host suggesting that one or more selective agents favor host specialization by the different lineages. In addition, we were able to morphologically separate individual lice from the two experimental lineages using discriminant function analysis. Furthermore, differences in the size of these louse lineages match differences in the size of their respective hosts, paralleling the strong correlation between parasite and host body size across the genus *Columbicola*. Together, these results suggest that selection in this cryptic species complex reflects selection across the whole genus, and that this selection, in part, contributes to the maintenance of host specialization.

KEY WORDS: **Birds, *Columbicola*, host specificity, morphology, parasites, selection.**

Host-reliant organisms, like parasites and phytophagous insects, are excellent systems for studying the role of selection in differentiation and speciation (Coyne and Orr 2004; Huyse et al. 2005). Parasites, in particular, can be highly specialized, both in the food resources they use (e.g., blood-feeding or feather-feeding) and in their host use—many parasites are highly host specific (Poulin 2007). In theory, each host species represents a novel selective environment for parasites, even in the absence of obvious geographic isolation (e.g., overlapping host ranges, Stireman et al. 2005). For example, the precise form of host defense may differ between host species, leading to different selective effects on parasites exploiting different hosts.

Host-specific parasites, especially those with simple, direct life cycles, are exposed primarily to the unique selective environment on the host, which may result in adaptation to that host (Lajeunesse and Forbes 2002). Adaptation, in turn, may reinforce specialization by increasing fitness on the native host, while generating reduced fitness on "wrong" hosts (Joshi and Thompson 1995; Gandon and Michalakis 2002). It is this trade-off between adaptation for one host species at the expense of being unable to exploit other host species that underlies the selective advantage of specialization (Joshi and Thompson 1995; Kassen 2002). For example, Katakura et al. (1989) described two closely related species of ladybird beetle (*Epilachna* spp.) that are specific to different host plants. The host plants have widely overlapping ranges; however, even when the two host plants are touching, the beetles remain specific to their native host, where their fitness is higher than on the nonnative host.

A successful host switch by specialized parasites has several possible outcomes. One possibility is the expansion of host use, that is, a reduction in specialization. The other possibility is that genetic isolation follows a host switch, and the new isolated population eventually becomes differentiated and speciates (Banks and Paterson 2005). This does not lead to a reduction in specialization, but to an increase in the number of specialized host-specific species. These outcomes are mediated by parasite transmission rate (Gandon and Michalakis 2002) and by the strength of the advantage conferred by specialization (Drés and Mallet 2002). If host species are not isolated, so that interhost transmission is common, then differentiation is less likely. However, large differences in the novel host environment or strong selection for specialization will encourage differentiation and speciation on the novel host, even in absence of host isolation (Kaltz et al. 1999; Drés and Mallet 2002; Criscione et al. 2005; McCoy et al. 2005; Stireman et al. 2005).

Compared to plant-herbivore systems, the relative importance of geographic isolation and host specialization in the speciation of animal parasites is poorly understood (Gandon et al. 1998; McCoy et al. 2002; Poulin 2007). This is true even for "permanent" parasites that complete their entire life cycle on the body of a single host individual, relying largely on a direct contact between host individuals for transmission. In some cases, such parasites show genetic variation between adjacent populations of the same host species, or even between host individuals within a single population. For example, Nadler et al. (1990) documented significant genetic differences between ectoparasitic chewing louse populations living on conspecific pocket gophers inhabiting burrows just a few meters apart. Although dispersal limitations undoubtedly play a role in genetic differentiation at this scale, host specialization may also be important (McCoy et al. 2002). Unfortunately, rigorous tests for an adaptive component to such genetic differentiation in animal parasites have been few and far between (Poulin 2007). The goal of our project was to test for a selective advantage to host specialization among divergent mitochondrial lineages present within a single described species of parasitic feather louse.

Feather lice (Phthiraptera: Ischnocera) are obligate, permanent ectoparasites that pass their entire life cycle on the body of the host. Most of the 2700 + species of lice that infest birds are quite host specific, being found, on average, on two species of hosts (Price et al. 2003). The generation time of most feather lice is less than a month (Marshall 1981). Given the much longer generation time of their hosts, it is reasonable to assume that many generations of lice can pass on a typical host individual. As might be expected, feather lice are highly specialized for life on birds, where they consume feathers and dead skin, and glue their eggs to feathers with a glandular cement. They have a simple life cycle with no intermediate hosts, and they transmit to new host individuals primarily during periods of direct contact between hosts, such as between parents and their offspring in the nest (Clayton and Tompkins 1994). Some feather lice are also capable of moving between hosts by hitchhiking phoretically on hippoboscid flies (Diptera: Hippoboscidae; Keirans 1975; Harbison et al. 2008). Hippoboscid flies are winged, blood-feeding parasites that are more mobile, and less host specific, than lice. This alternative means of transmission presumably offers these lice a path for host switching.

We studied the feather louse genus *Columbicola* (Ewing), which contains 88 described species, all of which are parasites of pigeons and doves (Columbiformes; Bush et al., in press). Most *Columbicola* are quite host specific, being found on an average of about two species of hosts. A notable exception is *C. macrourae* (Wilson), the least specific member of the genus, which is known from 15 different New World pigeon and dove species (Price et al. 2003). Although *C. macrourae* specimens from these different hosts represent a single morphospecies (Clayton and Price 1999), five mtDNA haplotype clusters have been documented from just half of the known *C. macrourae* hosts (Johnson et al. 2002; Clayton and Johnson 2003). Interestingly, these haplotype clusters are host specific and quite distinct, with up to 21% sequence divergence between clusters (Johnson et al. 2002), suggesting the presence of cryptic species (Bickford et al. 2007). Although host-associated genetic clades have been documented among other *Columbicola* species with multiple hosts, they are not ubiquitous. Indeed, two of the other less host-specific species in the genus (*C. gracilicapitus* and *C. adamsi*) have been documented from multiple hosts (three and five host species, respectively; Price et al. 2003) with no evidence of host-associated molecular differentiation (Johnson et al. 2007).

The apparent lack of morphological differentiation in *C. macrourae* from different hosts, combined with the striking genetic variation, suggests that physical isolation alone could be responsible for the genetic differentiation. However, several patterns in the genetic data indicate otherwise. To begin with, host use is not perfectly correlated with haplotype cluster. Some haplotype clusters have multiple, geographically overlapping hosts, yet show no evidence of genetic structure between these hosts (Johnson et al. 2002). This pattern implies that gene flow is still occurring between the louse populations on some host species. Furthermore, there is no evidence that *C. macrourae* has cospeciated with its Columbiform hosts (Clayton and Johnson 2003), which would be likely if the lice were truly completely isolated on their respective hosts. Rather, it seems that large-scale biogeographic patterns explain host use (Johnson et al. 2003, 2007). All the hosts used by *C. macrourae* are New World pigeons and doves with varying degrees of range overlap across much of the Americas (with the exception of the completely allopatrically isolated Galapagos Dove, *Zenaida galapagoensis*). Ancestral *C. macrourae* likely

acquired its various hosts by transmitting among, and establishing on, existing sympatric dove species in a stepping stone fashion, possibly via phoretic dispersal on hippoboscid flies (Clayton et al. 2004).

The evidence for multiple host switching events begs the question of whether the changes in host use have selected for adaptive changes in these lice. To test for an adaptive component linking genetic differentiation and host use, we asked three questions: (1) Will additional mtDNA sequencing and statistical analysis of *C. macrourae* specimens support the preliminary pattern of host specialization across genetically distinct clusters? (2) Will host-specific genetic lineages of lice within *C. macrourae* suffer a reduction in fitness on the "wrong" host? (3) Will a more detailed analysis of morphology reveal potentially specialized differences between these different lineages? Morphological similarities or differences between groups can be used to make inferences about the nature of selection (Simkova et al. 2002).

We began by sampling additional *C. macrourae* individuals from as many hosts as possible to evaluate the genetic structure in this species, and to test the host specificity of haplotype lineages. We then conducted a reciprocal transfer experiment using captive doves and their lice to test whether the host specialization of two common haplotype lineages confers a selective advantage. Specifically, we compared the fitness of lice on novel hosts to that of control lice transferred to parasite-free individuals of the native host (Tompkins and Clayton 1999; Gemmill et al. 2000; Bush and Clayton 2006). If there was an adaptive basis to host use, then a given lineage would be predicted to suffer reduced fitness on the novel host. Next, we used discriminant function analysis (DFA)to test for morphological differences between the two haplotype clusters involved in the reciprocal transfer experiment. Finally, we compared the morphological differences between these two haplotype clusters to known differences across other, conventional *Columbicola* species.

Materials and Methods

PHYLOGENETIC ANALYSIS

DNA was extracted from 17 *C. macrourae* specimens in the Price Institute for Phthirapteran Research (PIPeR) frozen tissue collection, as well as from 25 newly collected lice from wild-caught White-winged Doves (*Zenaida asiatica*) and Mourning Doves (*Zenaida macroura*, see below). Freshly collected lice were stored in vials of 70–95% ethanol at −80°C. To extract DNA, lice were decapitated, and the head and body were subjected to the Qiagen DNeasy Tissue kit protocol (Qiagen, Valencia, CA). This process facilitates penetration of the body cavity by extraction buffer while allowing for relatively easy "re-assembly" of lice as anatomical voucher specimens on microscope slides. Using PCR, we amplified the 379-bp section of the Cytochrome Oxidase I gene using the primer pair, H6625/L7005 (Hafner et al. 1994). Successful amplifications were visualized on agarose gel; the sample was purified using a Qiagen Purification Kit, then sent to the CORE Research Facility at the University of Utah for sequencing. We edited and aligned complimentary chromatograms using Sequencher 4.1 (GeneCodes). Additional *C. macrourae* sequences from Johnson et al. (2002), Clayton and Johnson (2003), and Johnson et al. (2007) were included in the analysis (Table 1). We also included sequences from *C. adamsi* and *C. extinctus*, which are nested within the *C. macrourae* haplotype complex (Johnson et al. 2007). We designated *C. waggermanni* as the outgroup because it is the basal member of the *extinctus* clade, a subgroup of *Columbicola* to which *C. macrourae* belongs (Adams et al. 2005; Johnson et al. 2007). The final dataset included 88 individual lice (GenBank Accession numbers pending). Sequences were aligned manually. Trees were reconstructed in PAUP* (Swofford 2001) using parsimony. Because we were primarily interested in the assignment of individuals to haplotype cluster, and not the higher level tree structure, more detailed phylogenetic analyses were not necessary. We conducted searches with 10 random addition replicates with TBR branch swapping. We ran 1000 bootstrap replicates to evaluate the robustness of clades.

RECIPROCAL TRANSFER EXPERIMENT

To test whether host specialization by different *C. macrourae* lineages has an adaptive component, we performed a reciprocal transfer experiment using lice from haplotype clusters 2 and 3 (Fig. 1), and their hosts, the White-winged Dove and Mourning Dove. White-winged Doves and Mourning Doves for the experiment were trapped in Lake Placid, Florida and Tucson, Arizona (federal permit #MB 836059; IACUC 02–09015). Birds were housed individually in 30 × 30 × 56 cm wire cages. The two species of doves were initially housed in separate animal rooms and were further subdivided into two groups: those for the reciprocal transfer experiment, and those for culturing lice to be used in the transfer experiment. Transfer experiment birds were kept in low-humidity animal rooms (< 30% r.h.) for ≥10 weeks to eliminate "background" louse infestations (Harbison et al. 2008). Feather lice "drink" by actively pumping water vapor from the air; at low humidity, they can no longer pump enough water to maintain equilibrium (Rudolph 1983). Culture birds were kept in humid rooms (> 60% r.h.), and were prevented from efficient preening using C-shaped plastic bits, inserted between the upper and lower mandibles of the bill. Bits spring shut slightly in the nostrils to prevent dislodging, but they do not damage the tissue. They create a 1–3 mm gap between the mandibles that impairs the forceps-like action of the bill required for efficient preening (Clayton et al. 2005). Bits do not interfere with feeding and they have no other apparent side effects (Clayton and Tompkins 1995).

Table 1. Louse samples included in phylogenetic tree.

Louse species[1]	Host species	Locality[2]	Louse ID	Host id no.	GenBank no.
1 – *Columbicola adamsi*	*Patagioenas nigrirostris*	Panama	Cosp.Conig.1.8.2003.14	VGR-189	EF678888
2 – *C. adamsi*	*P. picazuro*	Bolivia	Cotri.11.15.1999.3	CCW-956	EF678884
3 – *C. adamsi*	*P. picazuro*	Bolivia	Cosp.copic.1.31.05.2.a	CCW-956	FJ656426
4 – *C. adamsi*	*P. picazuro*	Bolivia	Cosp.copic.1.31.05.3.b	DGC 553	FJ656427
5 – *C. adamsi*	*P. plumbea*	Brazil	Cosp.Coplu.1.20.2003.2	JDW-461	FJ656428
6 – *C. adamsi*	*P. plumbea*	Guyana	Cosp.Coplu.10.19.1998.8	MJB-870	EF678885
7 – *C. adamsi*	*P. plumbea*	Guyana	Cosp.Coplu.4.24.1999.3	MBR-4355	EF678886
8 – *C. adamsi*	*P. plumbea*	Guyana	Cosp.coplu.1.31.05.4.a	MBR 4	FJ656429
9 – *C. adamsi*	*P. speciosa*	Campeche, Mexico	Coada.2.1.1999.5	CO-47	FJ656470
10 – *C. adamsi*	*P. speciosa*	Campeche, Mexico	Coada.10.19.1998.7	CO-43	AF278614
11 – *C. adamsi*	*P. speciosa*	Campeche, Mexico	Coada.3.1.1999.7	CO-43	EF678887
12 – *C. extinctus*	*P. fasciata*	Oregon	Coext.1.20.2003.1	none	EF678900
13 – *C. extinctus*	*P. fasciata*	Peru	Coext.10.12.1999.2	RCF1365	AY151010
14 – *C. extinctus*	*P. fasciata*	Oregon, USA	Cosp.Cofas.9.27.2000.4	none	EF678899
15 – *C. macrourae [1]*	*Geotrygon montana*	Guyana	Comac.3.1.1999.1	KSB-182	EF678892
16 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.3.1.1999.10	CO-8	AF414735
17 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.3.1.1999.8	CO-3	AF414736
18 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.3.1.1999.9	CO-1	AF414737
19 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.9.29.1998.1	CO-3	AF414738
20 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Cosp.Gemon.3.1.1999.4	CO-6	EF678891
21 – *C. macrourae [1]*	*G. montana*	Guyana	Cosp.Gemon.7.22.2004.2	KSB-182	FJ656430
22 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.gemon.1.10.05.1.a	GES 308	FJ656431
23 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.gemon.1.10.05.2.b	GES 309	FJ656432
24 – *C. macrourae [1]*	*G. montana*	Campeche, Mexico	Comac.gemon.1.10.05.3.c	GES408	AF414739
25 – *C. macrourae [1]*	*Leptotila plumbeiceps*	Campeche, Mexico	Cosp.Leplu.3.1.1999.6	CO-34	AF414740
26 – *C. macrourae [1]*	*L. plumbeiceps*	Campeche, Mexico	Cosp.plu.10.19.1998.4	CO-42	EF678889
27 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.10.14.1999.8	WT-49	AF414744
28 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.10.2.1999.12	WT-22	FJ656433
29 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.10.2.1999.5	WT-46	AF414749
30 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.9.14.1999.1	WT-22	AF414746
31 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.9.21.1999.7	WT-48	AF414748
32 – *C. macrourae [1]*	*L. verreauxi*	Peru	Cosp.Lever.11.15.1999.7	CCW-398	FJ656434
33 – *C. macrourae [1]*	*L. verreauxi*	Yucatan, Mexico	Cosp.Lever.2.1.1999.1	CO-25	AF414751
34 – *C. macrourae [1]*	*L. verreauxi*	Yucatan, Mexico	Cosp.ver.10.19.1998.2	CO-25	EF678890
35 – *C. macrourae [1]*	*L. verreauxi*	Texas, USA	Comac.10.14.1999.7	WT-49	AF414745
36 – *C. macrourae [2]*	*L. plumbeiceps*	Campeche, Mexico	Cosp.leplu.1.12.05.1.b	GES 410	FJ656435
37 – *C. macrourae [2]*	*L. verreauxi*	Texas, USA	Comac.2.1.1999.7	TX	AF414743
38 – *C. macrourae [2]*	*L. verreauxi*	Texas, USA	Comac.10.19.1998.3	Jul-98	AF414758
39 – *C. macrourae [2]*	*Zenaida asiatica*	Texas, USA	Comac.10.14.1999.5	WW-388	AF414754
40 – *C. macrourae [2]*	*Z. asiatica*	Texas, USA	Comac.10.2.1999.11	WW-392	AF414755
41 – *C. macrourae [2]*	*Z. asiatica*	Texas, USA	Comac.10.2.1999.4	WW-407	AF414757
42 – *C. macrourae [2]*	*Z. asiatica*	Texas, USA	Comac.2.1.1999.8	TX-358	AF414752
43 – *C. macrourae [2]*	*Z. asiatica*	Texas, USA	Comac.9.14.1999.8	WW-380	AF414756
44 – *C. macrourae [2]*	*Z. asiatica*	Texas, USA	Comac.9.29.1998.5	TX-358	EF678895
45 – *C. macrourae [2]*	*Z. asiatica*	Arizona, USA	Comac.Zeasi.6.08.04.6	JRM-WWD01	FJ656436
46 – *C. macrourae [2]*	*Z. asiatica*	Arizona, USA	Comac.Zeasi.6.08.04.7	JRM-WWD03	FJ656437
47 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.2.05.1Q1	JRM-WWD103	FJ656438
48 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.2.05.2Q1	JRM-WWD107	FJ656439
49 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.2.05.3Q1	JRM-WWD104	FJ656440
50 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.2.05.4Q1	JRM-WWD105	FJ656441
51 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.2.05.5Q1	JRM-WWD105	FJ656442

Continued

Table 1. Continued.

Louse species[1]	Host species	Locality[2]	Louse ID	Host id no.	GenBank no.
52 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.9.05.1Q1	JRM-WWD102	FJ656443
53 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.9.05.3Q1	JRM-WWD101	FJ656444
54 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.9.05.4Q1	JRM-WWD101	FJ656445
55 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.9.05.5Q1	JRM-WWD103	FJ656446
56 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.5.9.05.6Q1	JRM-WWD103	FJ656447
57 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.6.08.04.5	JRM-WWD106	FJ656448
58 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.8.1.05.1	JRM-WWD130	FJ656449
59 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.8.1.05.2	JRM-WWD130	FJ656450
60 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.8.1.05.3	JRM-WWD148	FJ656451
61 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.8.1.05.5	JRM-WWD121	FJ656452
62 – *C. macrourae [2]*	*Z. asiatica*	culture	Comac.Zeasi.8.1.05.6	JRM-WWD121	FJ656453
63 – *C. macrourae [3]*	*Z. macroura*	Texas, USA	Comac.10.14.1999.4	M-387	AF414760
64 – *C. macrourae [3]*	*Z. macroura*	Texas, USA	Comac.9.14.1999.5	M-379	AF414761
65 – *C. macrourae [3]*	*Z. macroura*	Texas, USA	Cosp.mac.10.19.1998.5	TX-358	FJ656471
66 – *C. macrourae [3]*	*Z. macroura*	Texas, USA	Cosp.Zemac.2.1.1999.9	TX-355	EF678898
67 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.2.05.7Q1	JRM-MD532	FJ656454
68 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.2.05.8Q1	JRM-MD533	FJ656455
69 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.9.05.8Q1	JRM-MD645	FJ656456
70 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.9.05.9Q1	JRM-MD645	FJ656457
71 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.9.05.10Q1	JRM-MD645	FJ656458
72 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.9.05.11Q1	JRM-MD644	FJ656459
73 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.Zemac.5.9.05.12Q1	JRM-MD646	FJ656460
74 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.zemac.6.08.04.3	JRM-MD530	FJ656461
75 – *C. macrourae [3]*	*Z. macroura*	culture	Comac.zemac.6.08.04.4	JRM-MD 533	FJ656462
76 – *C. macrourae [3]*	*Z. macroura*	Texas, USA	Comac.10.14.1999.2	M-394	AF278618
77 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.12.13.1999.7	none	EF678897
78 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.7.1.1999.2	1	AY594665
79 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.7.1.1999.3	3	AY594664
80 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.zegal.1.10.05.5.a	1	FJ656463
81 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.zegal.1.10.05.6.b	3	FJ656464
82 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Comac.zegal.1.10.05.8.d	5	FJ656465
83 – *C. macrourae [4]*	*Z. galapagoensis*	Galapagos, Ecuador	Cosp.zegal.1.31.05.8.b	none	FJ656466
84 – *C. macrourae [5]*	*P. subvinacea*	Brazil	Comac.11.15.1999.5	AA-415	AY151011
85 – *C. macrourae [5]*	*P. subvinacea*	Peru	Cosp.cosub.1.31.05.1.a	CCW 547	FJ656467
86 – *C. spp*	*P. oenops*	Peru	Cosp.Cooen.10.27.2003.5	REW-181	FJ656468
87 – *C.waggermanni*	*P. leucocephala*	Florida, USA	Cowag.11.15.1999.8	19-Jul-92	EF678901
88 – *C.waggermanni*	*P. leucocephala*	Florida, USA	Cowag.7.1.1999.7	19-Jul-92	FJ656469

[1]**Bracketed numbers after *C. macrourae* names indicate haplotype identity.**

[2]**"Culture" indicates lice sampled from laboratory culture birds. Haplotype 2 lice from culture birds originated in Florida or in Arizona, USA; haplotype 3 lice from culture birds originated in Florida, USA.**

Lice in the White-winged Dove culture were from birds captured in Florida and Arizona. Lice in the Mourning Dove culture were all from birds captured at sites in Florida, because Mourning Doves in Arizona do not host *C. macrourae* (J. R. Malenke, unpubl. data). All lice in culture were maintained on their native host species. We double-checked that the lice being cultured were indeed from the desired haplotype cluster, even though neither White-winged Doves nor Mourning Doves have previously been found with a "wrong" haplotype louse. Sixteen lice (predicted haplotype cluster 2) from 10 different White-winged Dove culture birds—from both Florida and Arizona—and nine lice (predicted haplotype cluster 3) from six different Mourning Dove culture birds (all Florida) were evaluated. Lice were removed from captive culture birds by exposing the bird to CO_2, thus immobilizing the lice, which could then be removed from the plumage by manually ruffling the feathers over a white sheet of paper (Moyer et al. 2002a). Lice were sampled twice: (1) shortly after capture, and (2) one year after capture, to ensure the long-term purity

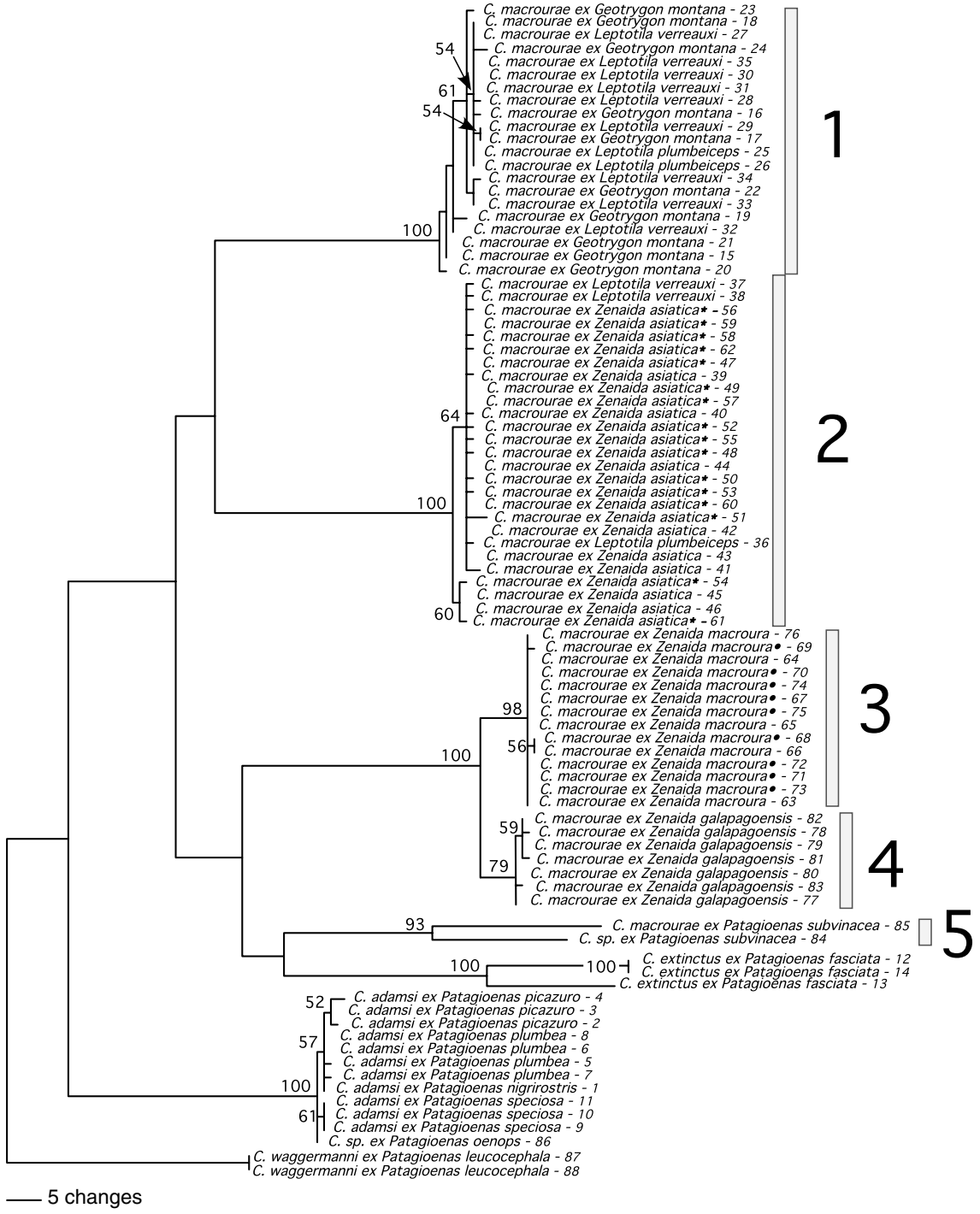

Figure 1. **Strict consensus parsimony tree based on an unweighted analysis of COI sequences. Branch length is scaled to the number of sequence changes (see scale bar). Bootstrap support of > 50% is indicated on nodes (1000 bootstrap replicates). Host common names (from Gibbs et al. [2001] and Johnson et al. [2001]) are: *Geotrygon montana* (Ruddy Quail-dove), *Leptotila verreauxi* (White-tipped Dove), *L. plumbeiceps* (Grey-headed Dove), *Zenaida asiatica* (White-winged Dove), *Z. macroura* (Mourning Dove), *Z. galapagoensis* (Galapagos Dove), *Patagioenas subvinacea* (Ruddy Pigeon), *P. fasciata* (Band-tailed Pigeon), *P. picazuro* (Picazuro Pigeon), *P. plumbea* [Vieillot] (Plumbeous Pigeon), *P. nigrirostris* (Short-billed Pigeon), *P. speciosa* (Scaled Pigeon), *P. oenops* (Peruvian Pigeon), and *P. leucocephala* (White-crowned Pigeon). *C. macrourae*, haplotype clusters are indicated by bars and the corresponding numbers 1–5 to the right of the tree. Haplotype clusters' identity numbers were first established in previous work (Johnson et al. 2002, 2003; Clayton and Johnson 2003). Louse species (mostly *C. macrourae*), and host species are identified for every specimen; numbers from 1 to 88 to the right of the names correspond to the individual information provided in Table 1. Asterisks are specimens sequenced from captive White-winged Dove culture birds; closed circles are specimens sequenced from captive Mourning Dove culture birds.**

of our cultures. DNA from these lice was extracted, sequenced, and subjected to phylogenetic analysis (as above), to confirm the mitochondrial lineage of the culture lice. All 16 lice sequenced from the White-winged Dove louse culture were members of the haplotype cluster 2 (two-tail Binomial test, $k = 0$; $P < 0.001$). All nine lice sequenced from the Mourning Dove louse culture were members of the haplotype cluster 3 (two-tailed Binomial test, $k = 0$, $P < 0.01$).

The reciprocal transfer experiment included 10 replicates. Each replicate consisted of two White-winged Doves and two Mourning Doves. One individual of each species was "seeded" with 25 adult White-winged Dove lice, whereas the other individual received 25 adult Mourning Dove lice. Lice were obtained from culture birds using the CO_2 method described above (Moyer et al. 2002a). The experiment was conducted in two trials separated in time by one year. The first trial consisted of four replicates; the second trial had six replicates. The timing and size of each trial was dictated by the availability of experimental animals.

During the course of the experiment, recipient White-winged Doves and Mourning Doves were housed individually in cages that were distributed randomly in a single animal room. The cages were separated by Plexiglas dividers to prevent contact between the feather tips of birds in adjacent cages, which could result in horizontal transfer of lice. The animal room was maintained at a relatively constant temperature (21°C), and the relative humidity was set at 70% r.h. Humidity varied more than temperature, especially between the two trials (years). The mean daily minimum r.h. in the first trial (four replicates) was 43%, whereas that in the second trial (six replicates) was 69% r.h. Birds were provided with ad libitum food and water for two months (about two louse generations [Martin 1934]). The birds were then euthanized, and their louse populations quantified using a body washing method that accounts for 99% of the lice (adults and nymphs) on a bird (Clayton and Drown 2001). Hence, our louse population data incorporated both the survival and reproductive components of parasite fitness. Populations were compared on native and novel hosts using paired nonparametric tests in JMP (version 5.1.).

MORPHOLOGY

To evaluate potential morphological differences, lice recovered at the end of the reciprocal transfer experiment were mounted on microscope slides using a standard protocol (Price et al. 2003). Up to three adult females and three adult males from each experimental bird seeded with native lice were mounted; however, some of the experimental louse populations were not large enough by the end of the experiment to provide the full complement of males and females. In addition, 10 female and 10 male lice were mounted from initial population samples preserved in 95% ethanol at the start of the experiment from both the White-winged Dove and Mourning Dove culture populations.

Mounted lice were examined using a Nikon DIC microscope with an ocular micrometer. The following measurements were taken from individual lice: head length, anterior plate median length, anterior plate width, temple width, prothorax width, metathorax width, total body length, number of setae on the median anterior section of the head, number of setae on abdominal tergite IX, number of setae lateral to genitalia (females only), antennal scape length (males only), and genital width (males only). These characters were chosen because they are traditional taxonomic characters used by Clayton and Price (1999) to discriminate between species of New World *Columbicola*. We omitted one setal character and two male genitalic characters, for which Clayton and Price (1999) could find no character variation, or could not score due to poor specimen quality.

These morphological characters were used as covariates in a DFA, which maximizes the variation between two designated groups (in this case, host identity). Individual lice missing one or more of the morphological characters were excluded, leaving a total of 12 female and 18 male lice from White-winged Doves, and 23 female and 22 male lice from Mourning Doves. Male and female lice were analyzed separately because they are sexually dimorphic.

Results

PHYLOGENETIC ANALYSIS

Our phylogenetic analysis of the mitochondrial COI sequences from 88 louse individuals, including the designated outgroup, recovered five genetically distinct haplotype clusters, three supported by bootstrap values $\geq$ 98%, and the remaining two by values $\geq$ 79% (Fig. 1). These haplotype clusters are consistent with those based on smaller numbers of specimens in Johnson et al. (2002) and Clayton and Johnson (2003), who showed up to 21% sequence divergence between lineages. Our more comprehensive sampling also revealed two new host records in the haplotype 2 clade: White-tipped Dove (*Leptotila verreauxi*) and Grey-headed Dove (*L. plumbeiceps*).

White-winged Doves, including the laboratory culture birds we sampled, hosted only haplotype cluster 2; Mourning Doves, including the culture birds, hosted only haplotype cluster 3. The lice sampled from the White-Winged Doves caught in Florida were not genetically distinguishable from the lice on White-winged Doves caught in Arizona (Fig. 1, Table 1). For these two hosts, we calculated the 95% upper confidence limit that our sampling could support using the exact binomial distribution method, that is, the largest percentage of "wrong" lice that could exist in the sampled host populations and still go undetected, given our sample sizes. With our sample of 24 lice from White-winged Doves, we were 95% confident of detecting any unpredicted haplotype present in the population with $\geq$11.8% frequency. With our sample of 14 lice

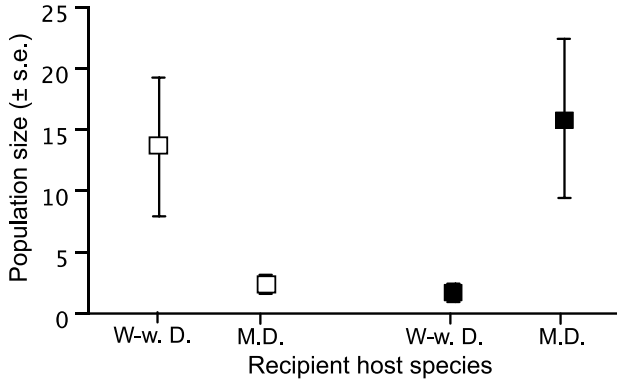

Figure 2. **Population sizes of *Columbicola macrourae* haplotype clusters 2 and 3 (from White-winged Doves and Mourning Doves, respectively) experimentally transferred to both native and novel hosts. W-w. D., White-winged Dove; M.D., Mourning Dove. Open squares (□) are the White-winged Dove cluster; closed squares (■) are the Mourning Dove cluster. Each haplotype cluster had significantly higher fitness on its native host, compared to the novel host (Wilcoxon signed rank test: White-winged Dove haplotype $P = 0.05$, $Z = -1.96$, df = 9; Mourning Dove haplotype $P < 0.05$, $Z = -2.19$; df = 9).**

from Mourning Doves, we were 95% confident of detecting an unpredicted haplotype with ≥19.2% frequency. Because the exact binomial distribution method is conservative in the event of zero negative cases, we consider these values to provide acceptable levels of uncertainty.

RECIPROCAL TRANSFER EXPERIMENT

Nymphal lice were present in all treatments at the end of the transfer experiment, indicating that the lice were both surviving and reproducing on native and novel host species. Louse population sizes varied between the two experimental trials (years) because of a temporary drop in ambient humidity in our animal rooms during the first year, which probably reduced reproductive rates (Moyer et al. 2002b). As a result, the effect size was smaller in the first year treatments, but the direction of change was the same. Because our treatments were balanced within each trial, the between year variation would not have biased our results.

Both *C. macrourae* mitochondrial lineages 2 and 3 had much larger populations on native hosts than on novel hosts at the end of the two-month experiment (Fig. 2; Fisher's combined probability test, $P < 0.005$, $\chi^2 = 13.02$, df = 4). The lice on novel hosts appeared to be heading for local extinction (Fig. 2).

MORPHOLOGY

Discriminant function analysis of transfer experiment specimens

The canonical DFA distinguished White-winged Dove lice from Mourning Dove lice on the basis of morphology, both for females (Wilk's Lambda $F = 51.6$, df = 10 23, $P < 0.001$) and males

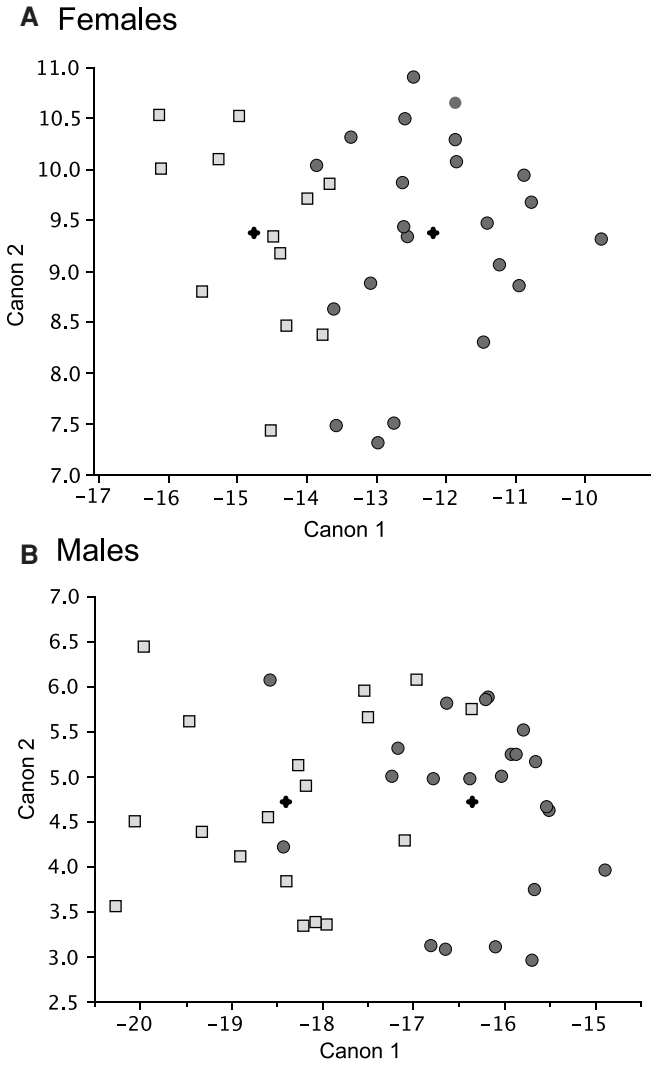

Figure 3. **Plot of the first and second canonical functions from a discriminant function analysis of *C. macrourae* morphological measurements. Open squares are White-winged Dove individuals (haplotype cluster 2); closed circles are Mourning Dove individuals (haplotype cluster 3). The small cross in each cloud of points represents the mean for that group. (A) Females. The values of Canon 1 for White-winged Dove female lice are significantly different from those of Mourning Dove female lice (Student's *t*-test, $t = -7.18$, df = 32, $P < 0.0001$). (B) Males. The values of Canon 1 for White-winged Dove male lice are also significantly different from those of Mourning Dove male lice ($t = -6.45$, df = 38, $P < 0.0001$).**

(Wilk's Lambda $F = 2.78$, df = 11 28, $P = 0.01$). The DFA for the females was able to correctly differentiate 91.2% of the specimens into host identity groups. The first canonical function was significantly different for female lice from White-winged Doves compared to Mourning Doves (Fig. 3A). The characters that contributed most to the first canonical function were, in decreasing order of effect: temple width, prothorax width, head length, metathorax width, and anterior plate length.

The DFA for the males was able to correctly differentiate 87.5% of the specimens into host identity groups, and the first canonical function was significantly different for lice from White-winged Doves and Mourning Doves (Fig. 3B). The characters that contributed most to the first canonical function of the males were, in decreasing order of effect: genital width, temple width, scape length, total length, and anterior plate length.

A post-hoc comparison of the temple width, which contributes strongly to both the female and male canonical functions, reveals that White-winged Dove lice (haplotype cluster 2) have significantly larger temple widths than Mourning Dove lice (haplotype cluster 3; 2 tailed t-tests: females: $t = 3.69$, df $= 43$, $P < 0.001$; males: $t = 4.00$, df $= 43$, $P < 0.0005$).

Discriminant function analysis of data from Clayton and Price (1999)

The reliance of traditional taxonomy on nonoverlapping character ranges may fail to recognize incipient or cryptic species. To investigate this possibility, we performed an additional DFA of *C. macrourae* from White-winged Doves and Mourning Doves using the raw morphological data from the *Columbicola* taxonomic revision by Clayton and Price (1999). The DFA could distinguish between female *C. macrourae* from White-winged Doves and those from Mourning Doves with 100% success (DFA model: Wilk's Lambda $F = 20.53$, df $= 11$, $P < 0.001$; first canon two-tailed t-test $t = 24.5$, df $= 16$, $P < 0.0001$). The DFA for males correctly classified 81% of the individuals; however, the model was not strong enough to discriminate males reliably (Wilk's lambda $F = 0.61$, df $= 11$, $P = 0.78$). Because these lice had already been mounted on microscope slides, their haplotype cluster designation could not be confirmed with DNA sequencing.

Discussion

Our mitochondrial tree contains sequences from 86 *Columbicola* individuals in the ingroup. The pattern of haplotype differentiation and host use revealed by this tree is consistent with the preliminary sampling efforts that motivated this study (Clayton and Johnson 2003; Johnson et al. 2003, 2007). All five mitochondrial haplotype clusters were present, and strongly supported by the bootstrap analysis. Our sampling effort nearly doubled the number of individuals in the ingroup ($n = 42$ new individuals). In addition, it increased the number of hosts to a total of 65 different host individuals and 13 host species. The breadth of sampling greatly reinforces the observation of the host specificity of these haplotypes, and minimizes the influence of potential sampling error.

Mitochondrial DNA data alone should typically not be used to distinguish species because the rapid rate of sequence evolution records only the maternal hereditary history (Wiens and Penkrot 2002; Olson et al. 2004). However, this rapid rate of evolution makes mtDNA ideal for detecting cryptic species (Wiens and Penkrot 2002; Bickford et al. 2007). When combined with additional lines of evidence, mtDNA has been shown to correctly distinguish species in groups as diverse as rotifers (Gomez et al. 2002) and tenrecs (Olson et al. 2004). Our tree contains more than mtDNA information; the significant congruence between the genetic differences, host use, and morphometric differences strongly suggests the presence of cryptic, host-specific species.

Our tree also reveals a few unexpected host records. Several lice from White-tipped Doves (*L. verreauxi*), as well as one from a Gray-headed Dove (*L. plumbeiceps*), fell out in haplotype cluster 2, which previously included only lice from White-winged Doves. White-tipped Doves, Gray-headed Doves, and White-winged Doves are not sister species, but their ranges do overlap. The presence of haplotype cluster 2 on all three host species raises the question as to whether these lice are really 100% host specific. The very low frequency of haplotype cluster 2 on two additional hosts may be due to "straggling," which is when lice disperse to a host on which they cannot actually establish a viable population. Straggling has been reported for other members of the *C. macrourae* complex that move from doves to predatory hawks, on which they do not establish viable populations (Whiteman et al. 2004). Interestingly, our field notes indicate that the White-tipped Dove individual with the unexpected haplotype cluster 2 louse in our study was mist-netted on the same day in the same net as several White-winged Doves, suggesting the possibility of transfer between birds hanging in nets.

Lice in haplotype cluster 1 also occurred on three host species: Ruddy Quail-dove (*Geotrygon montana*), White-tipped Dove (*L. verreauxi*) and Gray-headed Dove (*L. plumbeiceps*; Fig. 1). In this case, the multiple host records are probably not indicative of straggling, but represent a genuine lack of specificity. These multiple host associations are maintained across different collecting localities, for example Ruddy Quail-doves collected in both Mexico and Guyana have haplotype 1 cluster lice (Table 1). For these lice to maintain a single mitochondrial lineage across multiple hosts, they are presumably moving between host species. Lice can move between host individuals or species via a direct contact at shared feeding or roosting sites; they may also be transmitted between hosts at shared dust baths and nesting sites (Clayton et al. 2004). Interestingly, *C. macrourae* is also one of only two *Columbicola* species that has been documented phoretically hitching rides between potential hosts on parasitic hippoboscid flies (Couch 1962; Harbison et al. 2008).

A major goal was to test for a selective advantage to host specialization among divergent haplotype clusters present within a single described species of parasitic feather louse. Our reciprocal transfer experiment confirms that there is an adaptive component

to the host specialization of at least two mitochondrial lineages of *C. macrourae*. Differences between host species may facilitate divergence between louse populations on these different hosts. First, feather lice complete all stages of their life cycle, including reproduction, on the body of the host. Thus mating is more likely between individuals on the same host species than between different host species (Futuyma and Moreno 1988; Nosil 2002). This degree of inherent isolation may facilitate adaptive differentiation by reducing gene flow between populations under different selection pressures (Wiens 2004). Differences in selection between populations on different host species may further reinforce host specificity and isolation because individuals of intermediate phenotype may have reduced fitness on either host. This adaptive differentiation would further reduce gene flow between populations on different host species, eventually leading to speciation.

Traditional louse taxonomy usually attempts to distinguish species on the basis of nonoverlapping ranges in metric characters (Price et al. 2003). For this reason, it tends to be conservative, particularly when dealing with incipient species, or cryptic species (Poulin and Keeney 2008). In cases of very similar species, DFA can reveal differences that exist repeatedly across characters, and it can use these differences to discriminate predefined groups. Indeed, it is often used to expose cryptic species that are ecologically distinct (Yoder et al. 2000; Olson et al. 2004; Reed et al. 2004). Our DFA distinguished both female and male individuals of haplotype cluster 2 lice from haplotype cluster 3 lice. However, the covariates most responsible for differentiating females of clusters 2 and 3 were not identical to those differentiating males. These differences may reflect the influence of sexual selection (Simkova et al. 2002), especially given the importance of genital width and antennal scape length in males. Male *Columbicola* have bulky, dimorphic antennae with a large branch that is used to secure females during copulation. The role of sexual selection in the evolution of this species, and indeed the entire genus, is not well understood. The *C. macrourae* species complex may offer a new system to test the influence of sexual selection on divergence in a group with strong host associations.

Our reanalysis of the data in Clayton and Price (1999) also revealed clear differences in the morphology of haplotype clusters 2 and 3, at least in the case of females, even with much broader geographic sampling. (Clayton and Price [1999] measured lice from 10 White-winged Doves from five North American localities and lice from 15 Mourning Doves from 12 North American localities.) These results suggest that the morphological differences in our cultured lice are not merely a result of population differences at trapping localities, but that they reflect widespread patterns of variation.

These results may also have implications for the traditional taxonomic approach. Historically, host identity was used as a character in louse taxonomy and systematics. Over time, though, the importance of the host was downplayed as louse species were identified from multiple, sometimes unrelated host species. Clayton and Price (1999) grouped *C. macrourae* from 12 different columbiform host species as a single parasite species because the ranges of the various morphological measures were overlapping. However, our data suggest at least two of these host-specific populations are on unique evolutionary trajectories.

Cryptic parasite species are being described with increasing frequency as molecular fingerprinting of populations and species becomes more common (Poulin and Keeney 2008). To put our *C. macrourae* morphological differences into a broader macroevolutionary framework, we examined them in the context of Harrison's Rule (Harrison 1915), which states that parasite size increases with increasing host size. Harrison's Rule is important because it suggests that size-related selection influences parasite morphology across host taxa. Johnson et al. (2005) used size data from published taxonomic records to show that species of *Columbicola* and their columbiform hosts generally conform to Harrison's Rule. We found that *C. macrourae* from White-winged Doves versus Mourning Doves (Clayton and Price 1999) demonstrate the same positive relationship between host and parasite body size reported by Johnson et al. (2005, Fig. 4). The fact that White-winged Dove and Mourning Dove louse lineages fall within the general Harrison's Rule trend for different species of *Columbicola* and their hosts suggests that size-related selection may act on conspecific populations of lice in the same way that it acts on populations belonging to different species.

One selective agent responsible for the correlated size of *Columbicola* and their hosts is the main defense of birds against lice—preening with the bill (Clayton et al. 2005). *Columbicola* spp. escape preening by inserting their narrow bodies into the space between adjacent barbs of a host's wing and tail feathers. When Bush and Clayton (2006) experimentally transferred *Columbicola* from the native host to novel hosts of different sizes, the lice experienced a sharp reduction in population level fitness. For example, when *C. columbae* were transferred from Rock Pigeons (native host) to smaller novel hosts, louse population size was reduced dramatically. The reason for this reduction in fitness was that the lice were unable to hide in the smaller spaces between the feather barbs of small-bodied hosts, making the lice vulnerable to preening. When this experiment was repeated using birds in which preening was blocked, the fitness of lice on novel hosts was similar to that of lice on the native host. In a reciprocal experiment, *C. passerinae* experienced a sharp reduction in fitness when transferred to larger novel hosts from their native host, the Common Ground Dove *(Columbina passerina*). However, when the experiment was repeated using birds with blocked preening, the lice still had low fitness on novel hosts. The reason for the poor fitness of lice on large novel hosts with impaired preening is

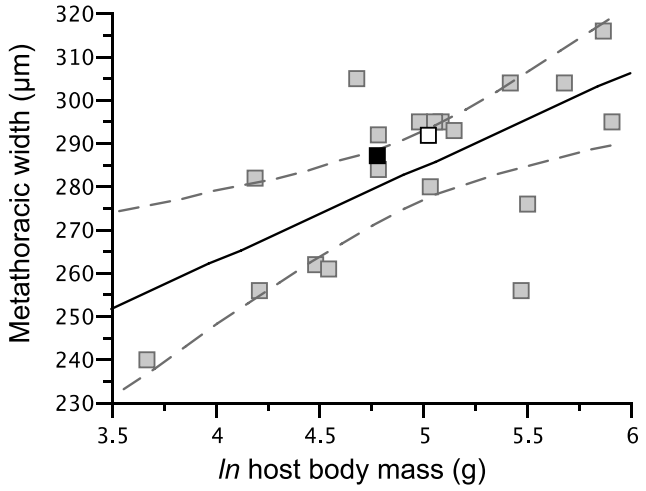

Figure 4. Harrison's Rule pattern of correlated host and parasite body size among 19 species of *Columbicola* and their hosts (gray boxes) reported by Johnson et al. (2005). Dashed lines show the 95% confidence intervals calculated for these data. The white box shows the mean metathorax width for 15 female *C. macrourae* from White-winged Doves (haplotype cluster 2); the black box shows the mean metathorax width for 10 female *C. macrourae* from Mourning Doves (haplotype cluster 3). Data for lice are from Clayton and Price (1999); host data are from Dunning (1993).

unclear (Bush and Clayton 2006). Nevertheless, preening selects for a match in host–parasite body size.

In summary, the molecular, experimental, and morphological comparative data reported herein help to create a more cohesive picture of the diversification of this group of parasites. Our phylogenetic results confirm the host specificity of divergent *C. macrourae* mitochondrial haplotype clusters, suggesting that this single morphospecies contains several cryptic species. The transfer experiments show that two of these lineages have much higher fitness on their native hosts than on novel hosts. Our results demonstrate that host specialization confers a selective advantage, which may buttress, or even spur the initial differentiation of cryptic species. We further reveal overlooked differences in the body sizes of these two lineages, differences that match small differences in host body size. These results, taken together, suggest that selection in this cryptic complex reflects selection across the genus *Columbicola*, maintaining host specialization.

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