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Do mosquitoes filter the access of *Plasmodium* cytochrome *b* lineages to an avian host?

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Abstract

Many parasites show fidelity to a set of hosts in ecological time but not evolutionary time and the determinants of this pattern are poorly understood. Malarial parasites use vertebrate hosts for the asexual stage of their life cycle but use Dipteran hosts for the sexual stage. Despite the potential evolutionary importance of Dipteran hosts, little is known of their role in determining a parasite's access to vertebrate hosts. Here, we use an avian malarial system in Panama to explore whether mosquitoes act as an access filter that limits the range of vertebrate hosts used by particular parasite lineages. We amplified and sequenced *Plasmodium* mitochondrial DNA (mtDNA) from *Turdus grayi* (clay-coloured robin) and from mosquitoes at the same study site. We trapped and identified to species 123 141 female mosquitoes and completed polymerase chain reaction (PCR) screening for *Plasmodium* parasites in 435 pools of 20 mosquitoes per pool (8700 individuals total) spanning the 11 most common mosquito species. Our primers amplified nine *Plasmodium* lineages, whose sequences differed by 1.72%–10.0%. Phylogenetic analyses revealed partial clustering of lineages that co-occurred in mosquito hosts. However PAN3 and PAN6, the two primary parasite lineages of *T. grayi*, exhibited sequence divergence of 8.59% and did not cluster in the phylogeny. We detected these two lineages exclusively in mosquitoes from different genera – PAN3 was found only in *Culex (Melanoconion) ocossa*, and PAN6 was found only in *Aedeomyia squamipennis*. Furthermore, each of these two parasite lineages co-occurred in mosquitoes with other *Plasmodium* lineages that were not found in the vertebrate host *T. grayi*. Together, this evidence suggests that parasite–mosquito associations do not restrict the access of parasites to birds but instead may actually facilitate the switching of vertebrate hosts that occurs over evolutionary time.

Keywords: access filter, avian malaria, host shift, host-parasite coevolution, life cycle, parasite diversity

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Introduction

Epidemiological disasters often occur when an infectious organism invades novel, immunologically naive host species (van Riper III *et al*. 1986; Lawson 1995; Hofmeyr *et al*. 2000). Such shifts occur when the factors that determine host range are altered. Thus, in order to understand the circumstances that lead to host range shifts we must first uncover what limits parasite-host range in the present time. The host range of a parasite can be defined most simply as the number of host species in which a parasite can successfully produce infective progeny (Matthews 1998). Although it is widely held that host ranges are kept small by coevolutionary arms races between host and parasite (Fry 1990; Lajeunesse & Forbes 2002), there are persistent generalist parasites (Ricklefs & Fallon 2002; Szymanski & Lovette 2005) and there is abundant

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documentation of parasites shifting to new hosts (Escalante *et al*. 1995; Waldenstrom *et al*. 2002; Ricklefs *et al*. 2004). Therefore, coevolution is insufficient to explain the wide variation in parasite host range size observed in nature. The coarsest determinants of host range have been proposed as two filters (Combes 2001). The host range of a parasite is made up of species that are (i) encountered by the parasite; and (ii) compatible with the parasite (i.e. low immune response, high resource value). In this paper, we explore the role of mosquitoes as a potential encounter filter that determines the access of avian malarial parasites (*Plasmodium* spp.) to a neotropical avian host, the clay-coloured robin *Turdus grayi*.

Recent studies of the ecology of avian malarial parasites have used DNA sequencing of the cytochrome *b* gene to identify parasites in blood samples of hundreds of bird species from both new and old world (Bensch *et al*. 2000; Fallon *et al*. 2003, 2004; Beadell *et al*. 2004; Szymanski & Lovette 2005; Hellgren *et al*. 2007; Ishtiaq *et al*. 2007). Although there is no consensus about how to define species with such data, these studies have revealed an enormous diversity of parasites perhaps on the same order as the number of bird species. A wide continuum of host ranges exists in this group of parasites and this trait has no phylogenetic pattern, i.e. specialist and generalist parasites may be close relatives (Ricklefs & Fallon 2002). Furthermore, comparison of host and parasite phylogenies indicates host-switching has been common in the history of the group of parasites: closely related parasites may be found in distantly related host species, and distantly related parasites can share a single host species. Thus, a paradox exists because parasites show fidelity to a group of hosts in ecological time but almost none in evolutionary time (Ricklefs *et al*. 2004). This begs the question of what factors restrict a parasite to its vertebrate-host range in ecological time when, in this group of parasites, host switching has been relatively common in history and the physiological capability to expand host range clearly exists.

Mosquitoes have the potential to act as an external factor that determines the access of malarial parasites to a pool of vertebrate hosts in ecological time but could change readily over evolutionary time, resulting in the changes seen in phylogenetic analyses of vertebrate-host use. Malarial parasites have a two-stage life cycle, with sexual reproduction occurring in a mosquito host and asexual reproduction occurring in a vertebrate host. Mosquitoes are thus critical components of this system for two reasons (i) because parasite sexual reproduction occurs in the mosquito, this life stage is integral to the reproductive isolation of parasite lineages; and (ii) behavioural and ecological aspects of mosquitoes (e.g. seasonality, geographical range, circadian patterns of behaviour) can determine the vertebrate hosts to which the parasites are exposed. Yet most recent studies of associations between avian malarial parasites and their vertebrate hosts have neglected the role of mosquitoes, primarily because of the logistical difficulties of assessing parasites' use of mosquito hosts. In this study, we explore the prevalence, diversity, and phylogeny of avian malarial parasites in common neotropical mosquito species and in a neotropical avian host, *T. grayi*, to test whether the vertebrate-host range of parasites is filtered by the ecology and behaviour of their invertebrate host.

Turdus grayi is a common tropical resident bird species in Central America. The population at our study site at the Smithsonian Tropical Research Institute in Gamboa, Panama, is regularly infected by two parasite lineages in the genus *Plasmodium*, PAN3 and PAN6 (unpublished data). Other avian species at this site are also infected with *Plasmodium* spp., though those patterns of host association are not well described to date. Using PCR and DNA sequencing of parasite cytochrome *b* sequences, we examine the distribution and phylogenetic relationships of *Plasmodium* lineages (including PAN3 and PAN6) in the 11 most common mosquito species in Gamboa. If PAN3 and PAN6 co-occur in a particular mosquito species, then this 'access filter' could explain their co-occurrence in *T. grayi*. If PAN3 and PAN6 occur in different mosquito species, the access filter is less likely to explain their co-occurrence in an avian host. The strongest evidence against a mosquito-based access filter would be if particular mosquitoes carry multiple *Plasmodium* lineages with only a subset of those lineages occurring in a particular avian host species, such as *T. grayi*.

Materials and methods

Study system

This study was conducted at the Gamboa Field Research Station of the Smithsonian Tropical Research Institute in central Panama. The habitat of the station includes grasslands and secondary humid tropical forest bordering the Panama Canal. *Turdus grayi*, the clay-coloured robin, is a year-round tropical resident and one of the most common species in the study area (Ridgely & Gwynne 1989).

The genus *Plasmodium* (Apicomplexa: Haemosporidia) is paraphyletic and includes parasites found in lizards and birds, which together form a sister group to the genus *Haemoproteus* (Bensch *et al*. 2000; Perkins & Schall 2002; Ricklefs & Fallon 2002; Beadell *et al*. 2004). The genus *Plasmodium* also includes the more distantly related group of parasites found in mammals, including those that cause malaria in humans (Escalante *et al*. 1995, 1998; Rathore *et al*. 2001). The development of techniques for the molecular identification of Haemosporidian parasites (including the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Hepatocystis*) has revealed a species diversity which was previously obscured by morphological similarities between

species (Bensch *et al*. 2000; Ricklefs & Fallon 2002; Beadell *et al*. 2004; Ishtiaq *et al*. 2007), though there remains the difficulty of reconciling the historical species concepts. All malarial parasites have a two-stage life cycle; the asexual stage occurs in a vertebrate host and the sexual stage in an invertebrate host in the order Diptera (flies, mosquitoes and relatives). Mosquitoes in the genus *Culex* are thought to be the most common hosts for avian *Plasmodium* parasites (Valkiûnas 2004) although species in the genera *Aedeomyia* (Crewe 1976; Gabaldon *et al*. 1981), *Aedes* (Eyles 1951) and others have also been identified either in natural or experimental studies.

Mosquito samples

Our goal was to sample host-seeking female mosquitoes that thus would have the potential to transmit *Plasmodium* to avian hosts. We collected mosquitoes over an eight-month period from August 2003 to March 2004, using eight Center for Disease Control (CDC) incandescent light traps, two CDC ultraviolet light traps, and two carbon dioxide arbovirus traps; the use of multiple trap types helps broaden the sampling of a mosquito community. Traps were set out bimonthly for 12 h each time (6 p.m–6 a.m) for a total of more than 2300 trap-hours. Following each 12-h trapping period, collection bags were removed from traps and placed in a freezer within two hours of trap closing. Individual mosquitoes were then sorted by sex and the females were sorted to species based on morphology. To avoid sequencing *Plasmodium* DNA from parasites picked up in blood meals but unable to develop (i.e. parasites incompatible with that particular mosquito host), visibly gravid females and mosquitoes with visible undigested blood meals were removed from the study. Individual mosquitoes were then put in pools of 20 individuals per cryotube and frozen until DNA extraction. Dr James Pecor of the US Army Walter Reed Biosystematics Unit confirmed species identification. Mounted specimen collections from this project have been deposited in the Natural History Museum at the University of Costa Rica and in the Entomology Department at the University of Panama.

DNA extraction and sequencing

DNA was extracted from pools of 20 mosquitoes each, following the procedure of Oskam *et al*. (1996). The procedure was modified by using a macerating centrifuge with ceramic beads for 45 s instead of grinding the mosquitoes with mortar and pestle. Mosquitoes were macerated in 800 μL fly lysis buffer (50 mM NaCl, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 7.4, 1% Triton X-100 and 10 mM DTT) followed by 3 h of incubation at 60 °C with 200 μg/mL proteinase K. The samples were then put through four freeze-thaw cycles (30 s at –80 °C in liquid nitrogen and 1 minute at 65 °C) to ensure the rupture of all membranes. Samples were centrifuged for 15 min at 12 000 × g and the supernatant was eluted and stored at –20 °C for PCR. The supernatant was thawed and diluted 1:5 in sterile, distilled water just prior to use in PCR.

We amplified a 360-bp section of the *Plasmodium* cytochrome *b* gene using the primer pairs (621) 5′-AAAAATACCY TTCTATCCAARTC-3′ and (983) 5′-CAYCCAATCCATAA TAAAGCAT-3′ modified from Richard *et al*. (2002) to be ambiguous at a total of three bases. This primer pair has been shown to amplify a wide range of cytochrome *b* sequences from old world and new world birds in the genera *Plasmodium* and *Haemoproteus* (Richard *et al*. 2002; Ricklefs & Fallon 2002). Amplifications were carried out in 50 μL reactions containing 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1 mM MgCl, 5.0 μL Qiagen PCR 10× buffer, 1.5 units of Taq polymerase (Qiagen) and 5 μL of diluted DNA. PCR was initiated by a 3-minute denaturing step followed by 35 cycles of 94 °C for 30 s, 50 °C for 40 s and 72 °C for 1 minute with a final extension step at 72 °C for 3 min. PCR products were visualized on a 2% agarose gel. Products that were faint were re-amplified by taking a small plug from the agarose gel, re-suspending the DNA in 100 μL distilled, sterile water at 65 °C and using 5 μL of this suspension in a second amplification; there were no apparent differences in parasite identity or diversity between samples that amplified strongly in the first PCR vs. those that required re-amplification. PCR products were cleaned in 2% agarose gels and sequenced directly on an MJ BaseStation Automatic sequencer (using the same forward and reverse primers used for amplification). For validation, all PCR products were sequenced in both directions. Sequences were edited and aligned in the program SEQUENCHER 4.1 (Gene Codes Corporation).

Phylogenetic analyses

Phylogenetic analyses of amplified cytochrome *b* sequences were carried out using maximum likelihood (ML) and neighbour joining (NJ) methods with the PAUP 4.0b10 software package (Swofford 2002). For the ML analysis we used MODELTEST 3.5 (Posada & Crandall 1998; using the script of B. Weir, http://www.rhizobia.co.nz/phylogenetics/modeltest.html) to compare the likelihood scores of trees (hierarchical log-likelihood tests) from 56 different models of nucleotide substitution. We determined that the best model, given the sequence data, included a six-step transition rate matrix and nucleotide base frequencies calculated from the data, a gamma shape parameter of 0.8874 and a proportion of invariable sites of 0.5984. This corresponds to a generalized time-reversible model of evolution with gamma-distributed and invariant sites (GTR + G + I). The tree generated with the above parameters had only a slightly higher log-likelihood score and an identical

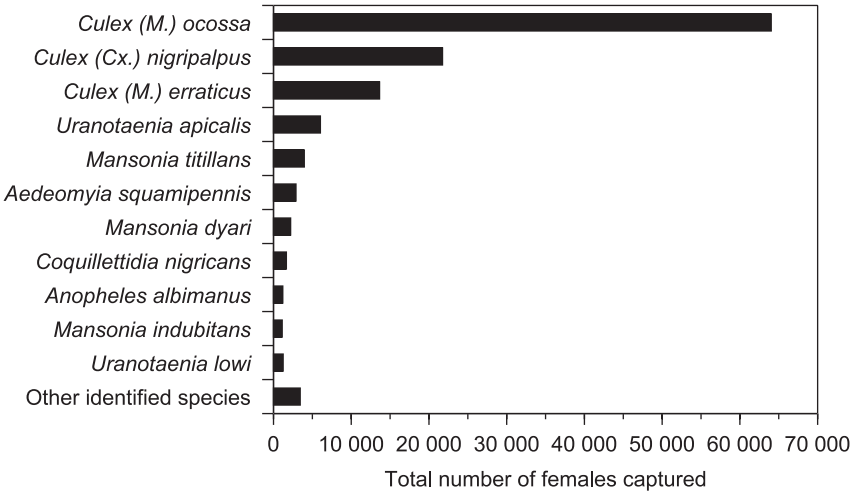

Fig. 1 The total number of female mosquitoes from the 11 most common species caught in traps from August 2003 through September 2004. Twenty additional species were identified in smaller numbers and were not PCR-screened for parasites. These included *Anopheles punctimacula* (*n* = 72), *An. triannulatus* (*n* = 853), *Coquillettidia venezuelensis* (*n* = 338), *Culex (Culex) coronator* (*n* = 118), *Cx. (Cx.) declarator* (*n* = 56), *Cx. (Cx.) quinquefasciatus* (*n* = 507), *Cx. (Melanoconion) conspirator* (*n* = 80), *Cx. (M.) interrogator* (*n* = 114), *Cx. (M.) seteki* (*n* = 281), *Cx. (M.) taeniopus* (*n* = 94), *Cx. (M.) tecmarsis* (*n* = 4), *Limathus durhami* (*n* = 13), *Mansonia flaveola* (*n* = 6), *M. pseudotitillans* (*n* = 75), *Aedes (Ochlerotatus) angustivittatus* (*n* = 110), *Aedes (Ochlerotatus) fulvus* (*n* = 12), *Aedes (Ochlerotatus) serratus* (*n* = 475), *Psorophora ciliata* (*n* = 163), *Sabethes cyaneus* (*n* = 13) *and Wyeomyia abebela* (*n* = 9).

topology to a model without invariable sites (GTR + *G*). The GTR is the most complex model of nucleotide evolution in the hierarchical analysis and allows for a unique value for each nucleotide transition rate. This level of complexity is warranted in this case because of the extreme AT bias in *Plasmodium* mitochondrial genes (Bahl *et al*. 2003), and GTR models have been used previously in phylogenetic analysis of malarial parasites (Perkins & Schall 2002). NJ analysis was carried out with distance measures from the ML parameters determined by MODELTEST 3.5 as described above. All parasites have been designated as *Plasmodium* by phylogenetic affinity to published avian *Plasmodium* cytochrome *b* sequences. Phylogenetic analysis was performed on the nine sequences amplified from screened mosquitoes in this study along with two previously published cytochrome *b* sequences of parasite species with known mosquito host species. These additional sequences were from *P. relictum* [National Centre For Biotechnology Information (NCBI) Accession No. AF069611] and *P. elongatum* (NCBI Accession No. AY099032) both of which are transmitted by *Culex* species in the subgenus *Culex* (Work *et al*. 1990; McConkey *et al*. 1996; Fonseca *et al*. 1998; Nayar *et al*. 1998). We plotted the number of novel parasite sequences identified against the number of individual mosquitoes screened in order to determine which mosquito species was associated with the greater number of parasite lineages and whether the cumulative number of parasite lineages associated with each mosquito species appeared to be approaching an asymptote.

Results

Identification of Plasmodium *cytochrome* b *lineages in pools of mosquitoes*

During the eight-month study period, we trapped a total of 128 713 female mosquitoes, of which 123 141 (95.7%) were identified to species. The remaining 5572 individuals could be identified only to subgenus (*n* = 5489 *Culex* (*Melanoconion*) sp., *n* = 78 *Culex* (*Culex*) sp., *n* = 5 *Haemagogus* spp.). Of 30 unambiguous species in the sample, 11 species comprised 97.2% of the fully identified individuals (Fig. 1). Using PCR with parasite-specific primers for cytochrome *b*, we screened a total of 435 pools of 20 mosquitoes each (8700 individuals total) from the 11 most common species collected. Twenty of the 435 pools screened were positive for parasite presence: 11 from *Aedeomyia squamipennis* and nine from *Culex* (*Melanoconion*) *ocossa*. A higher percentage of pools of *Ad. squamipennis* (11/39 = 28.2%) than *Cx. (M.) ocossa* (9/76 = 11.8%) were PCR-positive for parasite presence ($\chi^2 = 4.51$, d.f. = 1, $P = 0.034$). *Ad. squamipennis* has previously been reported as a host species for avian *Plasmodium* (Gabaldon *et al*. 1981). This is a first report of *Cx. (M.) ocossa* as a mosquito host for avian *Plasmodium.* The remaining nine species screened had no positive pools using this primer pair (Table 1). These included *Culex nigripalpus*, a species previously reported as host for avian *Plasmodium* in other parts of its range (Nayar *et al*. 1998).

Table 1 The mosquito species screened, the number of pools screened per species and the number of pools found to be PCR-positive for presence of parasites

Mosquito species	No. of pools screened (20 females per pool)	No. of pools PCR positive
Aedeomyia squamipennis*	**39**	**11**
Anopheles albimanus	10	0
*Culex (Cx.) nigripalpus**	63	0
Culex (M.) erraticus	51	0
Culex (M.) ocossa	**76**	**9**
Coquillettidia nigricans	32	0
Mansonia dyari	40	0
Mansonia indubitans	14	0
Mansonia titillans	52	0
Uranotaenia apicalis	32	0
Uranotaenia lowi	10	0

*Species previously identified as hosts for avian *Plasmodium* include *Ad. squamipennis* (Crewe 1976) and *Culex (Cx.) nigripalpus* (Nayar *et al*. 1998).

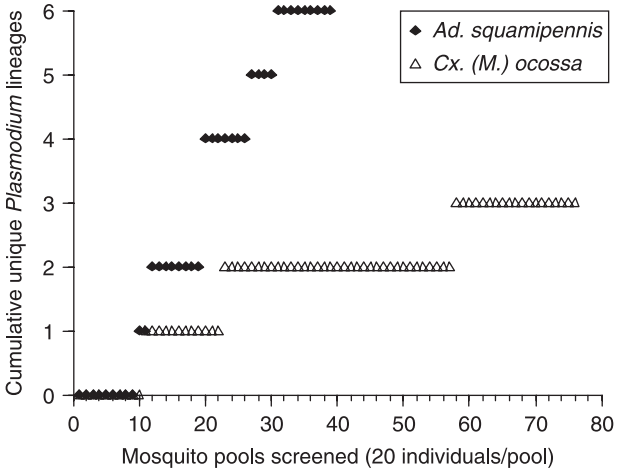

Fig. 2 The cumulative numbers of unique *Plasmodium* cytochrome *b* lineages sequenced from pools of individual mosquitoes of two mosquito species, *Aedeomyia squamipennis* and *Culex (Melanoconion) ocossa*.

Diversity and phylogenetic analysis of parasite cytochrome b *lineages*

The mosquito samples contained nine unique cytochrome *b* sequences of *Plasmodium* (designated PAN1–PAN9; GenBank accession nos. EU600217–EU600225), differing from one another by 1.72%–10% (uncorrected-p distance measure; Table 2). One pool of *Ad. squamipennis* yielded a sequence with double peaks indicative of mixed infection. The forward and reverse sequences were identical, and the pattern of double peaks corresponded perfectly to a mixed infection by PAN1 and PAN6, each of which had been found by itself in different pools of the same mosquito species.

Four of the nine *Plasmodium* lineages have also been found in birds at this study site GenBank accession nos. EU600226–EU6600230: two lineages (PAN1 and PAN5) were found when we conducted a preliminary screen of five individuals each of blue-grey tanager *Thraupis episcopus*, variable seedeater *Sporophila americana*, and red-legged honeycreeper *Cynanerpes cyaneus*; two other lineages, PAN3 and PAN6 were found frequently in a larger screening of *Turdus grayi* (unpublished data). We have designed more specific primers to detect PAN3 and PAN6 in *T. grayi* (unpublished data), and we have begun using these in conjunction with the degenerate primers used in this study of mosquitoes. To date, we have sequenced > 100 *Plasmodium*-positive individuals of *T. grayi*, and all but one of the infected birds contained only PAN3 or only PAN6 or a mixed infection of the two lineages, i.e. PAN3 and PAN6 are the common lineages in T. grayi, sometimes occurring together and sometimes separately (unpublished. data). In mosquitoes, PAN3 was found in two pools of *Cx. (M.) ocossa* and PAN6 was found in six pools of *Ad. squamipennis* (Table 3).

Table 2 Uncorrected-p distances between *Plasmodium* cytochrome *b* sequences amplified from two mosquito species, *Ad. squamipennis* and *Culex (Melanoconion) ocossa*. PAN2, 3 and 7 were identified in pools of *Cx. (M.) ocossa*. PAN1, 4, 5, 6, 8 and 9 were identified in *Ad. squamipennis*

PAN1	**PAN1**									
PAN2	0.081	**PAN2**								
PAN3*	0.099	0.024	**PAN3***							
PAN4	0.100	0.078	0.089	**PAN4**						
PAN5	0.097	0.075	0.095	0.017	**PAN5**					
PAN6*	0.086	0.075	0.086	0.037	0.040	**PAN6***				
PAN7	0.074	0.047	0.061	0.074	0.077	0.066	**PAN7**			
PAN8	0.061	0.068	0.082	0.086	0.083	0.070	0.056	**PAN8**		
PAN9	0.086	0.075	0.092	0.034	0.040	0.037	0.066	0.064	**PAN9**	
P. relictum	0.080	0.053	0.071	0.077	0.074	0.063	0.054	0.025	0.063	**P. relictum**
P. elongatum	0.080	0.066	0.072	0.089	0.086	0.069	0.066	0.040	0.074	0.017

*PAN3 and PAN6 are the *Plasmodium* lineages found in *T. grayi* (unpublished data).

Table 3 Number of pools of *Aedeomyia squamipennis* and *Culex (M.) ocossa* in which each *Plasmodium* lineage was detected

Plasmodium lineages	*Aedeomyia squamipennis*	*Culex (M.) ocossa*
PAN1*	*2*	*0*
PAN2	*0*	5
PAN3**	*0*	*2*
PAN4	*1*	0
PAN5*	*1*	*0*
PAN6**	*6*	*0*
PAN7	*0*	2
PAN8	*1*	0
PAN9	*1*	0

**denotes lineages found in extensive screen of *Turdus grayi* at this site (unpublished data); *denotes lineages found in preliminary screen of five individuals of three other bird species at this site (unpublished data).

A total of six unique *Plasmodium* lineages were found after screening 39 pools (780 individuals) of *Ad. squamipennis*, and three lineages were found in 76 pools (1520 individuals) of *Cx. (M.) ocossa*. In neither mosquito species does it appear that the rate of discovery of new sequences is decreasing significantly, i.e. approaching an asymptote, which would indicate that the total parasite sequence diversity is almost uncovered (Fig. 2). It is clear that the rate of discovery is higher in *Ad. squamipennis* than in *Cx. (M.) ocossa*.

Figure 3 shows the results of ML phylogenetic reconstructions of the nine sequences we found in mosquitoes in Panama and the two sequences from GenBank of *Plasmodium* species with known mosquito-host species. This analysis reveals phylogenetic clustering, although incomplete, among the parasites identified from the same mosquito species. Similar relationships were seen in a neighbour-joining tree. In both NJ and ML trees, PAN2 and PAN3, both from *Cx. (M.) ocossa*, are sister lineages with high bootstrap support (98% in both analyses). PAN7 clusters with these two lineages although with a bootstrap value less than 50%. Four of the parasites from *Ad. squamipennis*, PAN4, 5, 6 and 9, form a monophyletic clade with high bootstrap values in both NJ and ML (83 and 74%, respectively). However, PAN1 and PAN8, also from *Ad. squamipennis* pools, are found outside of this clade. PAN8 was found in a third grouping, in both NJ and ML phylogenies, with *P. relictum* and *P. elongatum*, both of which have *Culex (Culex)* species as hosts. By this measure, PAN2 and 3 are the closest sequences to PAN7 in the group. The uncorrected-p distances between the cytochrome *b* sequences amplified from mosquitoes are shown in Table 3.

Discussion

Sharing a vertebrate host but not sharing a mosquito host

The two *Plasmodium* parasites PAN3 and PAN6 are both found in *Turdus grayi* at Gamboa, Panama, sometimes co-occurring in the same individual bird. When we surveyed mosquitoes at Gamboa, we detected both PAN3 and PAN6 at this study site but in two different mosquito species – in fact, in different mosquito genera. The cytochrome *b* lineage PAN3 was found in two PCR pools of *Culex (Melanoconion) ocossa*, and PAN6 was found in six PCR pools of *Aedeomyia squamipennis*. These data imply that the co-occurrence of two parasite lineages in the same vertebrate host is not explained by access being provided by a shared mosquito host. This co-occurrence in an avian host of congeneric malarial parasites transmitted by different mosquito genera contrasts with the most widely studied *Plasmodium*-mosquito-vertebrate host system, namely human malaria. In that system, multiple parasite species that share a vertebrate host are transmitted by mosquitoes in the same genus or even the same species (Oaks *et al*. 1991).

Because our sampling of mosquito species was not exhaustive, it is important to consider what pattern of host distribution might exist but remain undetected. Therefore, we address the likelihood that, with further screening of mosquito individuals and species, PAN3 and PAN6 would be found in additional mosquito species and, possibly, in the same mosquito species. PAN3 was found in *Cx. (M.) ocossa*, which belongs to one of the most speciose mosquito groups in the neotropics (Pecor *et al*. 1992; Hutchings *et al*. 2005). We trapped but did not screen four other (less common) species of *Cx. (Melanoconion)* (Fig. 1), and additional species of this subgenus have been collected in the study area (Heinemann & Belkin 1978); thus the opportunity certainly exists for PAN3 to infect multiple *Culex (Melanoconion)* species in this geographical region. PAN6 was found in *Aedeomyia* which, in contrast to *Culex*, is monotypic in the Americas, having only a single species, *Ad. squamipennis*, found throughout much of Central and South America. It is possible that *Ad. squamipennis* is the only host for PAN6, because it would be an unprecedented taxonomic leap for a parasite to include *Aedeomyia* and other genera in its Dipteran-host range. For instance, human malaria species are transmitted by different mosquitoes in different geographical regions, but all are members of the genus *Anopheles* (Oaks *et al*. 1991; Donnelly *et al*. 2002; Krzywinski & Besansky 2003). Similarly, in avian malarial systems the vectors of *Plasmodium forresteri*, *P. elongatum*, *P. juxtanucleare, P. hermani* and *P. kempi* are all members of the same subgenus, *Culex (Culex)* (Christensen *et al*. 1983; Telford *et al*. 1997). Therefore, we believe that because PAN3 and PAN6 were identified in different mosquito genera, they will not be found to co-occur in

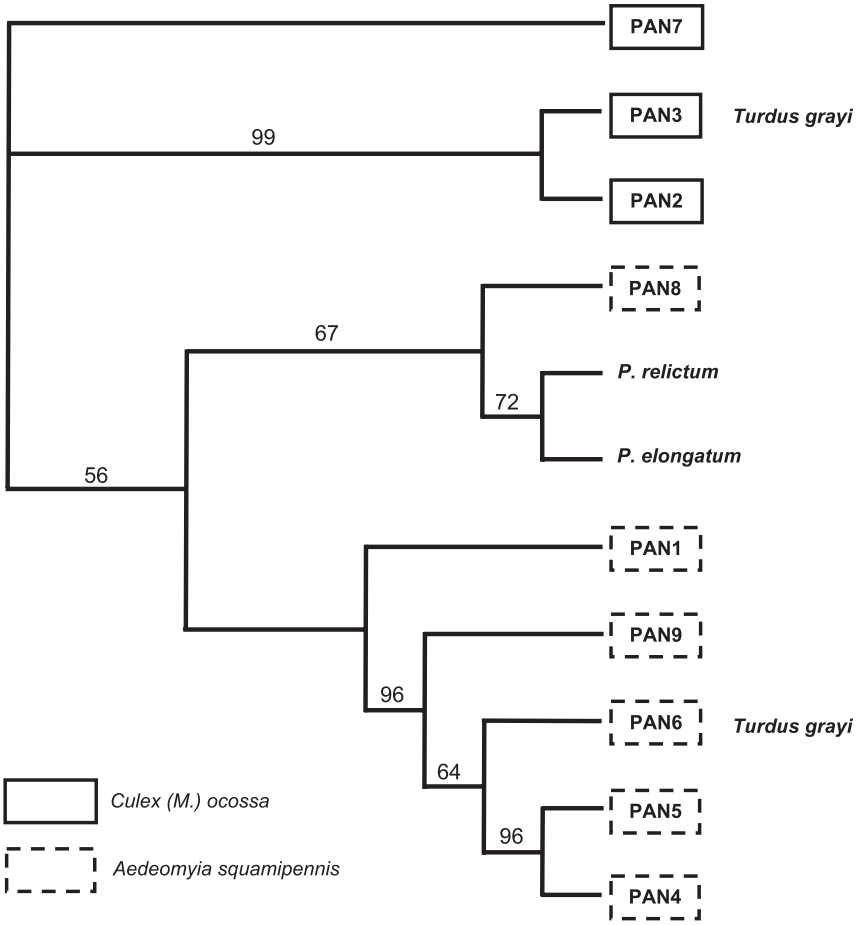

Fig. 3 Maximum likelihood tree of nine *Plasmodium* cytochrome *b* sequences obtained from pools of mosquitoes from our Gamboa site and of published sequences of *P. relictum* and *P. elongatum* (both transmitted by *Culex* (*Culex*) sp.). PAN3 and PAN6 are the two lineages commonly found in *Turdus grayi*. Likelihood parameters were determined using MODELTEST 3.5 and correspond to the GTR + G + I model with nucleotide frequencies determined by the data. Relationships were similar in a neighbour-joining tree.

mosquito species regardless of how much additional sampling is conducted.

An important implication of finding PAN3 and PAN6 exclusively in different mosquito species is that the parasites themselves should then be considered different species, rather than haplotypes of the same species. This group of parasites undergoes sexual recombination and reproduction in the mosquito; if they do not encounter one another during the sexual stage then they must be considered reproductively and evolutionarily isolated units. The implication that PAN3 and PAN6 are indeed reproductively isolated is consistent with three lines of genetic evidence:

1 The genetic distance between PAN3 and PAN6 (8.2% uncorrected-p) is much higher than the average pairwise distances between *Plasmodium* cytochrome *b* sequences from many other avian studies (Ricklefs *et al*. 2005).

2 This genetic distance is also greater than the average cytochrome *b* distance among three most closely related human malarial parasites that are known to be different species: *P. vivax*, *P. malariae* and *P. ovale* (Escalante *et al*. 1998).

3 Work with avian *Plasmodium* has shown that lineages with cytochrome *b* distances as little as 1% exhibit no nuclear gene recombination, meaning that these lineages are reproductively isolated (Bensch *et al*. 2004).

Finding two lineages exclusively in different mosquitoes safely implies reproductive isolation, but finding two lineages that share a mosquito host does not necessarily mean that they are the same species. For example, some lineages of *Plasmodium* overlapped in mosquito hosts but had different avian hosts and appeared to represent genetically distinct lineages: PAN6 (present in *T. grayi*) and PAN5 (absent in *T. grayi*) differed by 4.02% genetic distance despite both being found in *Ad. squamipennis*. Likewise, PAN3 (present in *T. grayi*) and PAN2 (absent in *T. grayi*) differed by 2.37% genetic distance despite both being found in *Cx. (M.) ocossa.* The finding of closely related parasite lineages sorting into different avian-host species in a community of birds has also been reported elsewhere (Ricklefs *et al.* 2005). This highlights the need for multiple lines of evidence when considering host-parasite coevolution and parasite taxonomy.

Diversity and phylogenetic relationships of parasites found in mosquitoes

Despite using only a single primer pair we found a surprising diversity of parasite lineages in only two mosquito species. Three lineages were found in *Cx. (M.) ocossa* and six additional lineages in *Ad. squamipennis*. Both the number of unique parasite sequences and the percentage of infected pools were higher in *Ad. squamipennis* than in *Cx. (M.) ocossa*. On average, each pool that we sampled yielded approximately 0.23 new sequences in *Ad. squamipennis* and 0.05 in *Cx. (M.) ocossa*, suggesting that in total *Ad. squamipennis* harbours many more parasites than *Cx. (M.) ocossa*. Given that four of the nine sequences recovered in this study were sequenced only once it is likely that many more sequences would be uncovered with further screening of mosquito pools. This is despite the fact that many mosquitoes were negative for *Plasmodium* in our PCR screens. This low rate of infection is consistent with studies based on microscopic examination of mosquito salivary glands, which have found infection rates as low as 1.9% for avian *Plasmodium* (Reeves *et al*. 1954) and < 1% for human *Plasmodium* (Charlwood *et al*. 2003, Shililu *et al*. 2004). This raises the methodological point that we amplified *Plasmodium* DNA from whole mosquitoes rather than from salivary glands. If any of the amplified parasite lineages were unable to develop into infective sporozoites in these mosquitoes (as has been seen in some studies of experimental infections, e.g. Grieco *et al*. 2005), then hosts would not be exposed to them. This possibility does not change the fact that PAN3 and PAN6 were found exclusively in mosquitoes of different genera, and it also seems unlikely that none of the seven other lineages was capable of developing into sporozoites.

Phylogenetic analysis of parasite sequences recovered from pools of mosquitoes showed that the majority of parasites found in a mosquito species exhibit a degree of monophyly but that this monophyly is incomplete because distantly related parasites can also be found in the same mosquito host. In *Ad. Squamipennis*, four of the six unique *Plasmodium* sequences formed a monophyletic clade that was highly supported. Similarly in *Cx. (M.) ocossa*, PAN2 and PAN3 are each other's closest relatives. Finally, *P. relictum* and *P. elongatum*, the additional two *Plasmodium* species that have been found in *Culex (Culex)* mosquitoes, also cluster together although with relatively lower support. Finding a clade of related but reproductively isolated *Plasmodium* lineages in a single mosquito species raises interesting questions about the dynamics of speciation in this system, because it suggests the possibility that speciation could have occurred sympatrically. Furthermore, the occurrence of a clade of related parasites in a single mosquito species is very unlike host associations of these parasites in birds, where there is less congruence between bird and parasite phylogenies (Ricklefs *et al*. 2004). This suggests that parasites are more restricted to their mosquito hosts than to their vertebrate hosts.

We now return to the original question of whether parasites that share a vertebrate host do so because of an encounter filter imposed by their mosquito host. Two lines of evidence from this study suggest that parasite–mosquito associations do not restrict the access of parasites to birds but rather seem to be the mechanism that facilitates the switching of avian hosts that we know has occurred in the evolutionary history of this system. First, parasite lineages found in *T. grayi* were found in different mosquito species. Second, the two parasite lineages of *T. grayi* were found in their respective mosquito species with several other parasite lineages that were not found in *T. grayi*. This implies that the distribution of parasites in *T. grayi* is not a simple reflection of the lineages that are afforded access to *T. grayi* by mosquitoes. Instead, vertebrate hosts appear to be frequently exposed to a diverse array of *Plasmodium* lineages, only some of which are regularly successful at inhabiting a particular vertebrate species. Thus, the frequent encounter that mosquitoes seem to create between apparently incompatible hosts and parasites may be the mechanism for *Plasmodium*'s switching of vertebrate hosts, rather than mosquitoes acting as an access filter that prevents such switching.

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