THESIS

CROSS-SPECIES TRANSMISSION OF A FELINE HAEMOPLASMA FROM DOMESTIC TO WILD CATS

Submitted by

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ABSTRACT

CROSS-SPECIES TRANSMISSION OF A FELINE HAEMOPLASMA FROM DOMESTIC TO WILD CATS

Disease poses a substantial threat to rare species worldwide. Spillover of multi-host pathogens from domestic to wild species may occur when a) closely related domestic and wild species co-occur and b) the high-density domestic species acts as a maintenance host with transmission to the rarer wild relative. Wild and domestic felids are susceptible to many of the same pathogens and co-exist near the interface of natural and developed landscapes. Our study evaluates cross-species transmission and host-switching of *Mycoplasma haemominutum* (*Mhm*), an erythrocytic bacterial parasite, between free-ranging domestic cats and nine wild felid species. We took a multi-pronged approach to evaluate transmission mechanisms by combining field surveys, modeling, model selection, pathogen genotyping and phylogenetic analyses. Our models consisted of *a priori* hypotheses of transmission pathways designed to predict observed prevalence data and were parameterized by site- and species-specific information. Using an information-theoretic approach we show that transmission via direct host contact is the most parsimonious hypothesis, which we then validated with genetic analyses. We traced transmission pathways by genotyping part of the bacterial 16S rRNA gene in 60 positive blood samples from domestic cats (n=19), bobcats (n=24), and pumas (n=17) from our extensive sampling efforts in California and Colorado for a total of 73 novel isolates (including co-infected samples). We combined these data with 49 previously described

sequences from GenBank, and carried out a partitioned Bayesian phylogenetic analysis reconstructing cross-species transmission events of *Mhm* on a global scale. The phylogenetic analysis validated our best-fitting models by showing that while *Mhm* is largely host-specific, cross-species transmission has occurred primarily in one direction following the trophic network from lower to higher levels. Our results are consistent with transmission by direct contact as the major mechanism for pathogen transfer of *Mhm*, and suggest that wild felids worldwide may be at risk for pathogen spillover from domestic cats.

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INTRODUCTION

Spillover of multi-host pathogens from domestic to wild animals may pose a substantial risk to rare species worldwide, leading to sudden and unpredictable population declines and extinctions (Daszak 2000; Viggers et al. 1993; Woodroffe 1999). Spillover may occur when a) closely related domestic and wild species co-occur and b) the high-density domestic species acts as a maintenance host with transmission to the rarer wild relative. Over the last several decades, multiple high-profile spillover events have occurred, including rabies outbreaks in African wild dogs (*Lycaon pictus*), bat-eared foxes (*Otocyon megalotis*), and Ethiopian wolves (*Canis simensis*) (Gascoyne et al. 1993; Maas 1993; Sillero-Zubiri et al. 1996); toxoplasmosis in marine mammals including sea otters (*Enhydra lutris*), Pacific walrus (*Odobenus rosmarus*), California sea lions (*Zalophus californianus*), and multiple species of dolphins and seals (Fayer et al. 2004); bovine tuberculosis in brushtail possum (*Trichosurus vulpecula*), European badger (*Meles meles*), American bison (*Bison bison*), African buffalo (*Syncerus caffer*), Marsh antelope (*Kobus lechwe*) and white-tailed deer (*Odocoileus virginianus*) (De Lisle et al. 2002); and Canine Distemper Virus in African lions (*Panthera leo*) (Roelke-Parker et al. 1996). In each of these epizootics, pathogen transfer from domestic to wild animals was implicated, as 90% of pathogens infecting domestic dogs and cats infect multiple host species (Cleaveland et al. 2001).

Wild felids are of particular conservation concern because most species are threatened, endangered, or experiencing population declines (IUCN 2015). Millions of domestic cats occur worldwide, roaming the edges of an ever-expanding human footprint (Churcher and Lawton 1987; Coleman and Temple 1993a; Crooks and Soulé 1999; O'Brien

et al. 2012). Wild felids are genetically and physiologically closely related to domestic cats and thus share susceptibility to many of the same bacterial, viral, and protozoal agents (Bevins et al. 2012; O'Brien and Yuhki 1999). High-profile spillover events from domestic to wild cats are uncommon, but have caused major population declines in rare felids in recent decades. In 1988, an epizootic outbreak of feline panleucopenia virus (FPV) in Florida killed 11 of 18 radio-collared bobcats (*Lynx rufus*) over a three-month period (Wassmer et al. 1988). In 2006, an outbreak of Feline Leukemia Virus (FeLV) in critically-endangered Iberian lynxes (*L. pardinus*) killed 6 of 14 FeLV-infected lynxes (Meli et al. 2009); this virus has also been documented in endangered Florida panthers (Brown et al. 2008). Other viral and bacterial disease agents shown to infect multiple felid host species include *Bartonella* spp., *Toxoplasma gondii*, Feline Immunodeficiency Virus (FIV), Feline Calcivirus, and the haemotropic mycoplasmas, including *Mycoplasma haemofelis* (*Mhf*), *Mycoplasma turicensis* (*Mtc*), and *Mycoplasma haemominutum (Mhm*) (Bevins et al. 2012; Chomel et al. 1996; Franklin et al. 2007; Meli et al. 2009; Sykes et al. 2008).

The haemotropic mycoplasmas ('haemoplasmas') are a recently characterized group of parasites widely distributed in wild and domestic cats (Tasker 2010; Willi et al. 2005). These gram-negative, wall-less bacteria bind to the surface of erythrocytes, causing chronic infection, although pathogenicity is dependent on the haemoplasma species and the underlying health of the infected individual (Tasker 2010). Prevalence varies between geographic areas and cat populations (e.g. feral v. pet, sick v. healthy), and haemoplasmas often occur as double or triple infections (Barker and Tasker 2013). *Mycoplasma haemominutum* (*Mhm*) is a more recently recognized haemoplasma (Neimark et al. 2001), usually presenting subclinically with generally higher prevalence than other

haemoplasmas (Tasker 2010). The chronic nature of *Mhm* infection combined with its high prevalence, low pathogenicity, and diminutive genome renders it a useful model organism in the study of felid transmission dynamics.

Haemoplasma transmission, however, is poorly understood, typical of newly recognized pathogens. Initially, transmission was presumed to be vector-borne on account of phenotypic similarity to vector-borne Rickettsial organisms (Neimark et al. 2001), and because of the co-occurrence of mycoplasmas within tick (Fyumagwa et al. 2008) and flea vectors and the blood of their domestic hosts (Barrs et al. 2010; Shaw et al. 2004; Woods et al. 2005). However, laboratory-based infection attempts have proven inconclusive, and only transient infection of *M. haemofelis* has been demonstrated through haematophagous activity (Woods et al. 2006). *Mhm* DNA has been isolated from the salivary glands and saliva of domestic cats, implicating social grooming and aggression as possible means of direct transmission (Dean et al. 2008). Museux et al. (2009) simulated social and aggressive transmission mechanisms by infecting domestic cats with *Ca. M. turicensis* subcutaneously, orally and oronasally, but of these, only subcutaneous inoculations with infected blood successfully transmitted infection. In sum, these results suggest intraspecific aggression and interspecific predation may play a major role in haemoplasma transmission.

Compelling genetic evidence suggests domestic cats are the source of the global distribution of haemoplasmas in wild felids because of high sequence identity between wild- and domestic-derived isolates, and substantial geographic overlap among similar isolates (Willi et al. 2007). While domestic cats may be the source of haemoplasma dispersion at a global scale, frequent high prevalence among wild felids suggests

subsequent intraspecific transmission and relaxed barriers to cross-species transmission (Willi 2007). Hereafter, we use the term "cross-species transmission" to refer to any instance of interspecific transmission, whether or not the infection was perpetuated in the new host. Given the global depth and breadth of *Mhm* prevalence, we assume that cross-species transmission may result in onward transmission within the new host species ('host-shift'), depending on the unique ecological circumstances of the novel host.

We hypothesized that *Mhm* is primarily host-specific, but that cross-species transmission and/or host-switching would be demonstrable through phylogenetic analyses of *Mhm* DNA sequences. We predicted that patterns of interspecific transmission would primarily follow the trophic network from lower to higher levels, providing support for direct transmission of *Mhm* and predation as a mechanism for pathogen spillover from domestic to wild cats. Our study evaluates transmission mechanisms and dynamics of *Mhm* by combining field surveys, modeling, model selection, genotyping of the 16S rRNA bacterial gene detected in host blood, and Bayesian phylogenetic analyses with ancestral host-state reconstruction. We conclude that cross-species transmission of *Mhm* is occurring through direct host contact, and that domestic cats are spreading haemoplasmosis to their rarer, wild relatives on a global scale.

METHODS

Study populations, sample collection and processing

North American pumas, bobcats, and domestic cats were sampled from four sites spanning two states: California and Colorado (Figure 1).

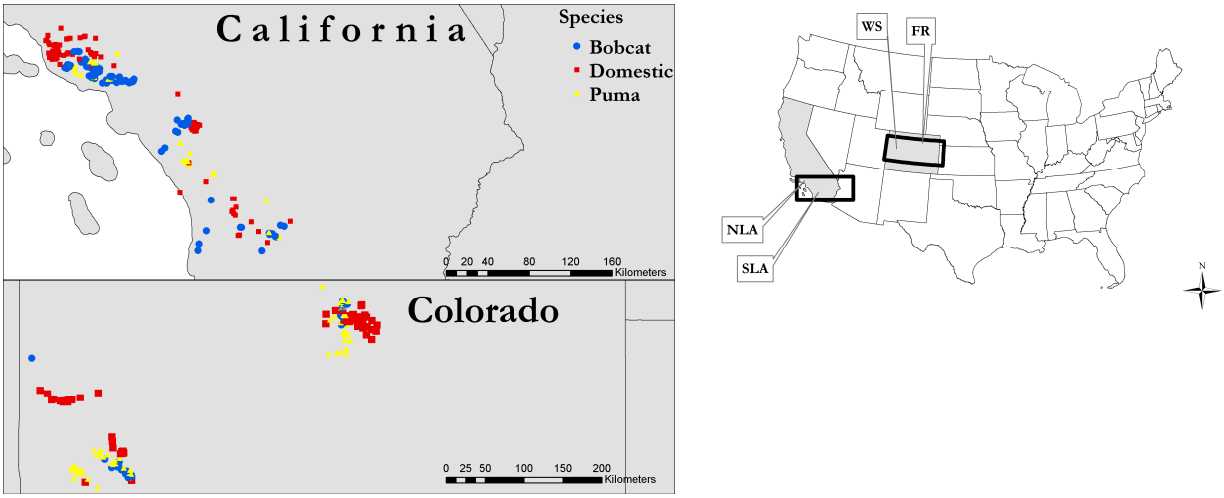


Figure 1. North American capture locations of pumas, bobcats, and domestic cats. California locations include Ventura and Los Angeles counties north of the City of Los Angeles (NLA), and Orange, San Diego, and Riverside counties south of the City of Los Angeles (SLA). Colorado capture locations include the Uncompahgre Plateau on the Western Slope (WS) of the Rocky Mountains near the city of Montrose, and the Front Range (FR) east of the Rocky Mountains near the city of Boulder.

California sites comprise two regions, divided by the City of Los Angeles. The area north of Los Angeles (NLA) includes Ventura County and some of Los Angeles County; the region south of Los Angeles (SLA) includes Orange, San Diego, and Riverside counties. Capture locations include urbanized landscapes as well as natural areas in the Santa Monica (NLA), Santa Ana (SLA) and Cuyamaca Mountains (SLA). California sites experience a warm, dry Mediterranean climate with vegetation communities consisting of coastal California sage scrub, chaparral, coastal and riparian woodlands, and annual grasslands.

Colorado sites include rural areas on the western slope (WS) of the Rocky Mountains near the cities of Montrose and Grand Junction, and the front range (FR) of the Rocky Mountains, immediately adjacent to Boulder, CO. The Colorado climate is cooler than California and semiarid, with vegetation comprising coniferous woodlands (WS: pinyon-juniper; FR: ponderosa-douglas fir) interlaced with aspens.

Samples were collected within each site over a 2-3 year period, with the majority collected between 2001-2012. Samples from bobcats and pumas were obtained from collaborators in conjunction with ongoing research (see Carver et al. 2016). For a subset of hunter-killed bobcats, thoracic fluid was collected in lieu of serum (Carver et al. 2012). Domestic cats were sampled opportunistically from free-ranging individuals on admission to shelters, or through Trap Neuter Release clinics in close proximity to bobcat and puma trapping locations. Blood, serum, and oral swab samples were collected from each individual after live-trapping (bobcats) or darting (pumas) and anesthetization (Logan and Sweanor 2001). In the field, blood and serum samples were stored in EDTA and serum-separating tubes, and subsequently refrigerated at 4°C or kept on ice until return to the lab. They were then temporarily frozen at -20°C and later transferred to -80°C until screening for pathogen exposure. All samples were collected in compliance with guidelines and protocols approved by the collection agency and their associated animal care committees.

Pathogen Screening

Infection with *Mhm* was assessed via DNA extracted from red blood cells and characterized through real-time PCR assays following protocol established by Jensen et al. (2001). This protocol is sensitive and mycoplasmal species-specific, and has been used extensively in studies of feline haemoplasmas (Wardrop et al. 2016; Willi et al. 2006; Willi

et al. 2005). In total, we tested 716 puma (n = 157), bobcat (n = 257), and domestic cat (n = 302) samples, of which 239 tested PCR-positive for *Mhm* (Table 1).

Table 1. Occurrence of *M. haemominutum* infection (*I*) among feral domestic cat, bobcat and puma samples (*N*) from southern California and Colorado as determined by PCR detection of rRNA16s sequences. Also shown are proportion of males (*m*, estimated from sample collection) and species densities (*d*, per kilometer, estimated from literature). See Table 2 for parameter definitions.

Site	Species	*N*	*I* (%)	*m*	d	Source	Location of source literature
North Los Angeles California	Domestic	74	22 (29.7)	0.62	35.2	Dabritz et al. (2006)	Moro Bay, CA
	Bobcat	179	85 (47.5)	0.54	0.21	Riley el al. (2010)	Santa Monica Mountains, CA
	Puma	32	20 (62.5)	0.72	0.011	Beier & Barett (1993)	Santa Ana Mountains, CA
South Los Angeles California	Domestic	56	8 (14.3)	0.54	35.2	Dabritz et al. (2006)	Moro Bay, CA
	Bobcat	20	13 (65.0)	0.75	0.23	Riley el al. (2010)	Santa Ana Mountains, CA
	Puma	10	5 (50.0)	0.70	0.011	Beier & Barett (1993)	Santa Ana Mountains, CA
Western Slope Colorado	Domestic	59	9 (15.3)	0.47	9.1	Warner (1985), Coleman & Temple (1993b), Hubbs (1951)	Rural CA, WI, Il
	Bobcat	25	8 (32.0)	0.56	0.194	Lewis et al. (2015)	Uncompahgre Plateau, CO
	Puma	46	21 (45.7)	0.35	0.022	Lewis et al. (2015)	Uncompahgre Plateau, CO
Front Range Colorado	Domestic	56	3 (5.4)	0.50	35.2	Dabritz et al. (2006)	Moro Bay, CA
	Bobcat	15	7 (46.7)	0.67	0.192	Lewis et al. (2015)	Boulder County, CO
	Puma	59	38 (64.4)	0.42	0.032	Lewis et al. (2015)	Boulder County, CO

Modeling of transmission mechanisms

We summarize the samples screened for *Mhm* infection and major population characteristics used for modeling of transmission mechanisms in Table 1. Estimates of species- and site-specific densities were obtained from published reports

and confirmed as representative by regional experts. Of the three felid species, densities of the domestic cat were least well-known and general estimates of urban edge and rural densities were compiled from published studies across the U.S. (Table 1). We used maximum likelihood estimation for graphical presentation of infection prevalence (± 95% CIs, see Figure 2).

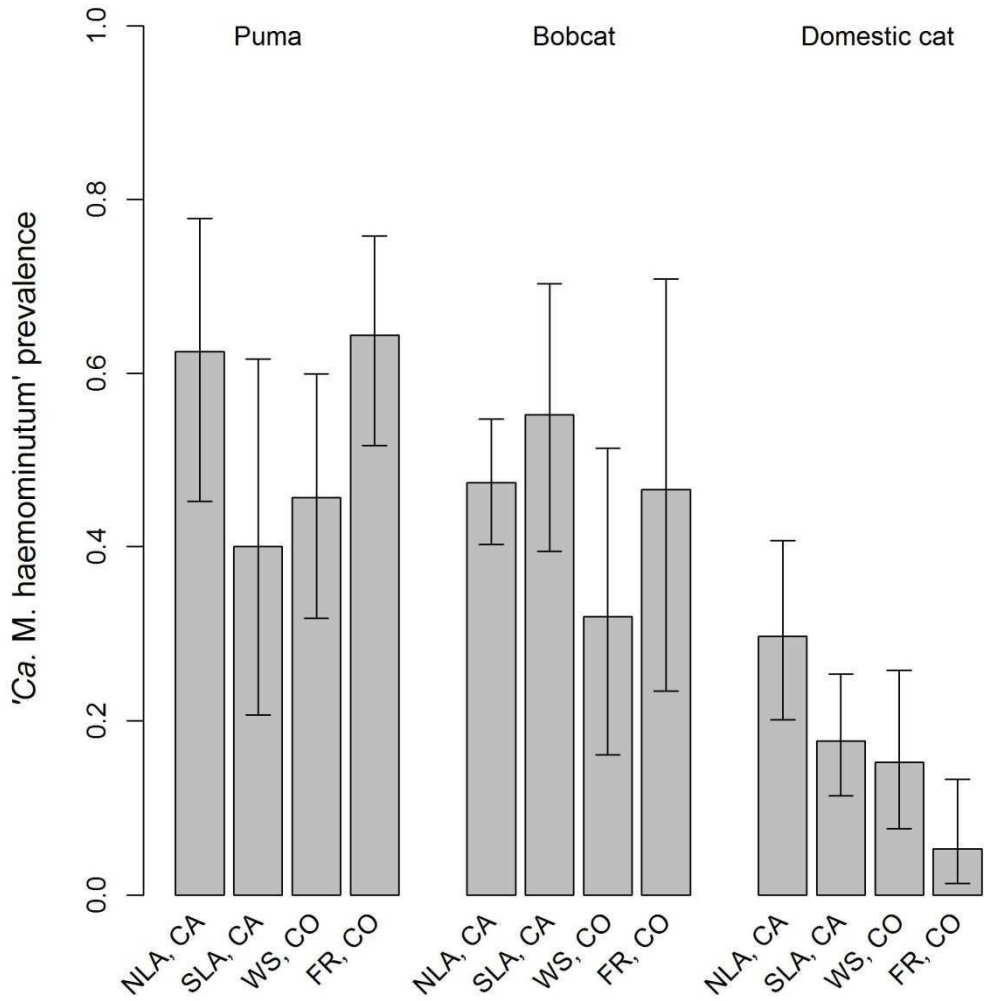


Figure 2. Prevalence (maximum likelihood estimates ± 95% CIs) of domestic cat, bobcats and puma with *M. haemominutum* infection across the study locations north and south of Los Angeles (NLA, SLA) California, and Western Slope (WS) and Front Range (FR) Colorado.

To evaluate potential mechanisms of intra- and inter-specific *Mhm* transmission among wild and domestic felids we created *a priori* hypotheses/models of transmission scenarios to predict observed prevalence (I/N, Table 2) of *Mhm* in California and Colorado. Transmission scenarios were characterized by algebraic expressions incorporating species- and site-specific parameters derived from the literature and/or determined by the authors (Table1, Table 2). The models are reasonable simplifications of possible transmission scenarios, given the limited information available on haemoplasma ecology and contact networks among felids. Seasonal- and age-specific differences in infection were not modeled because small sample sizes were insufficient to evaluate this confidently for some species at some sites.

Intraspecific transmission through social contact (1a, Table 2) assumes transmission by social interactions (e.g. grooming) and low-level aggressive encounters that may result from social interactions. Social contact hypotheses may include transmission by host-specific arthropod vectors, but evidence suggests this is unlikely for all three felid species (Currier 1983). Intraspecific transmission by aggressive encounters (1b, Table 2) occurs typically via male-biased interactions. We assumed intraguild predation routes could only occur in certain directions, i.e. predation of domestic cat by bobcat or puma and predation of bobcat by puma only (2a-d, Table 2). We are unaware of predation of bobcat by domestic cat or predation of puma by bobcat or domestic cat; we expect such events to be extremely rare. Vector-borne transmission was assumed to take place via generalist vectors (3a, Table 2), which could include ticks, fleas or mosquitoes. We also assumed the possibility of some directionality in vector-borne transmission, i.e.

Table 2. Algebraic expressions representing possible modes of *M. haemominutum* transmission. Expressions within each transmission mode further categorized (1-3) to describe intra- and inter-specific transmission (see Table 1 and Appendix 1).

Intra- and inter-specific transmission modes	Categories for each transmission mode		
	1	2	3
1. Intra-specific transmission			
1a. Social contact	$\Theta_{scD} d_D$	$\Theta_{scB} d_B$	$\Theta_{scP} d_P$
1b. Aggressive encounters	$\Theta_{agrD} m_D d_D$	$\Theta_{agrB} m_B d_B$	$\Theta_{agrP} m_P d_P$
2. Inter-specific transmission			
2a. Aggressive encounters between puma & domestic cat	$\Theta_{prPD} \frac{I_D}{N_D} d_D$		
2b. Aggressive encounters of bobcat & puma with domestic cat	$\Theta_{prPD} \frac{I_D}{N_D} d_D$	$\Theta_{prBD} \frac{I_D}{N_D} d_D$	
2c. Aggressive encounters of puma with domestic cat & bobcat	$\Theta_{prPD} \frac{I_D}{N_D} d_D$		$\Theta_{prPB} \frac{I_B}{N_B} d_B$
2d. Aggressive encounters of bobcat & puma with domestic cat, & puma with bobcat	$\Theta_{prPD} \frac{I_D}{N_D} d_D$	$\Theta_{prBD} \frac{I_D}{N_D} d_D$	$\Theta_{prPB} \frac{I_B}{N_B} d_B$
3. Intra- & inter-specific transmission			
3a. Generalist vector	$\Theta_v d_D$	$\Theta_v d_B$	$\Theta_v d_P$
3b. Bobcat & puma acquire domestic cat vectors	$\Theta_{vD} d_D$	$(\Theta_{vD} + \Theta_{vB}) d_B$	$(\Theta_{vD} + \Theta_{vP}) d_P$
3c. Bobcat & puma share vectors & acquire domestic cat vectors	$\Theta_{vD} d_D$	$(\Theta_{vD} + \Theta_{vBP}) d_B$	$(\Theta_{vD} + \Theta_{vBP}) d_P$
3d. Domestic cat acquire bobcat & puma vectors	$(\Theta_{vD} + \Theta_{vB} + \Theta_{vP}) d_D$	$\Theta_{vB} d_B$	$\Theta_{vP} d_P$
3e. Domestic cat acquire shared bobcat & puma vectors	$(\Theta_{vD} + \Theta_{vBP}) d_D$	$\Theta_{vBP} d_B$	$\Theta_{vBP} d_P$
3f. Environmental	$\Theta_{eD} (d_D + d_B + d_P)$	$\Theta_{eB} (d_D + d_B + d_P)$	$\Theta_{eP} (d_D + d_B + d_P)$

$_D$ = domestic cat; $_B$ = bobcat; $_P$ = puma; N = number of samples tested; I = number of infected samples; d = site-specific density of species per km^2; m = proportion of samples that are male; Θ_{sc} = transmission from social contact; Θ_{agr} = transmission from aggressive encounters; Θ_{pr} = transmission from predation; Θ_v = vector-borne transmission; Θ_e = environmental transmission

bobcats and puma having host-specific or shared vectors and acquiring infected host-specific vectors from domestic cats; domestic cats acquiring host-specific vectors from bobcats and pumas; and domestic cats acquiring shared vectors from bobcats and pumas (3a-e, Table 2). For environmental transmission (3f, Table 2) we assumed species-specific acquisition of environmental fomites from items such as substrate, carcasses, prey, and scat (for scent/territory marking).

Fit of *a priori* hypotheses to species-specific prevalence data among sites and estimation of unknown parameters (Θ) was estimated by maximum likelihood, based on binomial distributions. In each case *a priori* hypotheses were transformed as the inverse logit to constrain estimates of prevalence between zero and one. Best-fitting models were then identified using model selection and weighting procedures based on Akaike's information criterion (AIC). Owing to some model uncertainty (Table 3, Appendix 1), we calculated the relative importance of all transmission mechanisms over the entire model set (Burnham and Anderson 2002) using random forest analysis (Breiman 2001). Because traditional variable importance weight calculations are typically used with regression models and balanced model sets, we chose random forest analysis to allow for greater flexibility when using custom-built equations (Cutler et al. 2007). Importantly, we note that estimates of Θ in these models encompass much cumulative information for the felid populations, such as contact rates, transmission probabilities, seasonality of dynamics,

Table 3. Model selection of best fit (ΔAIC < 2) *a priori* hypotheses for intra- and inter-specific *M. haemominutum* transmission (I/N). See Table 2 for expressions, categories, and parameter definitions.

A priori hypothesized transmission	K	-2LOG(L)	AIC	ΔAIC	Weight (%)	Cumulative weight (%)
Aggression (1b), puma predate domestic (2a), domestic acquire shared bobcat & puma vectors (3e)	6	72.97	84.97	0.00	13.74	13.74
Aggression (1b), puma predate domestic (2a), bobcat & puma share vectors & acquire domestic vectors (3c)	6	73.99	85.99	1.02	8.27	22.00
Aggression (1b), puma predate domestic (2a), generalist vector (3a)	5	76.04	86.04	1.07	8.05	30.05
Aggression (1b), puma predate domestic & bobcat (2c), generalist vector (3a)	6	74.27	86.27	1.30	7.16	37.22
Aggression (1b), puma predate domestic & bobcat (2c), bobcat & puma share vectors & acquire domestic vectors (3c)	7	72.62	86.62	1.65	6.02	43.24
Aggression (1b), domestic acquire shared bobcat & puma vectors (3e)	6	74.74	86.74	1.77	5.68	48.92
Aggression (1b), bobcat and puma acquire domestic cat vectors (3b)	6	74.77	86.77	1.80	5.58	54.49
Aggression (1b) & generalist vector (3a)	4	78.86	86.86	1.89	5.34	59.84
Aggression (1b), bobcat & puma share vectors & acquire domestic vectors (3c)	4	78.86	86.86	1.89	5.34	65.18

environmental determinants of dynamics, vector abundance and species composition (for Θ_v), and the frequency of male aggression (for Θ_m).These scaling variables are necessary to define and parameterize these models/hypotheses but are not immediately biologically meaningful in and of themselves. All analyses were conducted in R (v.3.0.2; www.r-project.org) using the stats, stats4, rpart and randomForest packages.

Genotyping by sequencing of Mhm isolates

Sample selection

Of the 239 *Mhm*-positive samples, we sequenced a subset of 82 individuals for our phylogenetic analyses. We excluded animals co-infected with multiple *Mycoplasma* species (e.g. *M. haemominutum* and *M. haemofelis*) and preferentially selected samples from which DNA had been extracted for prior studies. We aimed for a roughly equal distribution of pumas, bobcats, and domestic cats from each study area. We ultimately excluded samples that failed to amplify, resulted in unreadable sequences, or were multiply-infected with > 2 unique *Mhm* genotypes. Our final data set of *Mhm* sequences included 73 novel *Mhm* sequences from 60 North American domestic cats (n=19), bobcats (n=24), and pumas (n=17). Thirteen North American individuals were co-infected with 2 *Mhm* strains. In addition, we incorporated 49 previously described sequences from GenBank for domestic (n=38) and wild felid species (n= 12) globally (Table 4; Figure 3).

Table 4. All samples used throughout analyses. 'Newly characterized samples' were used for modeling transmission mechanisms; all samples were used for phylogenetic analyses. FR = front range; WS = western slope; NLA = north of Los Angeles; SLA = south of Los Angeles. New samples will be updated with GenBank accession numbers upon submission.

Newly Characterized Samples

Animal ID	No. Samples	Host Species	Capture Location	Region
X1030; X1217; X1325	3	*F. catus*	USA	CO-FR
X1499; X596; X587*	3	*F. catus*	USA	CO-WS
X1001; X1062; X1209; X1314; X706; X1315*; X702*	7	*F. catus*	USA	CA-NLA
X1000; X1140; X1239; X1503; X1126*; X672	6	*F. catus*	USA	CA-SLA
X1288; X1313; X1316; X1328; X1363	5	*L. rufus*	USA	CO-FR
X947; X364*; X937*	3	*L. rufus*	USA	CO-WS

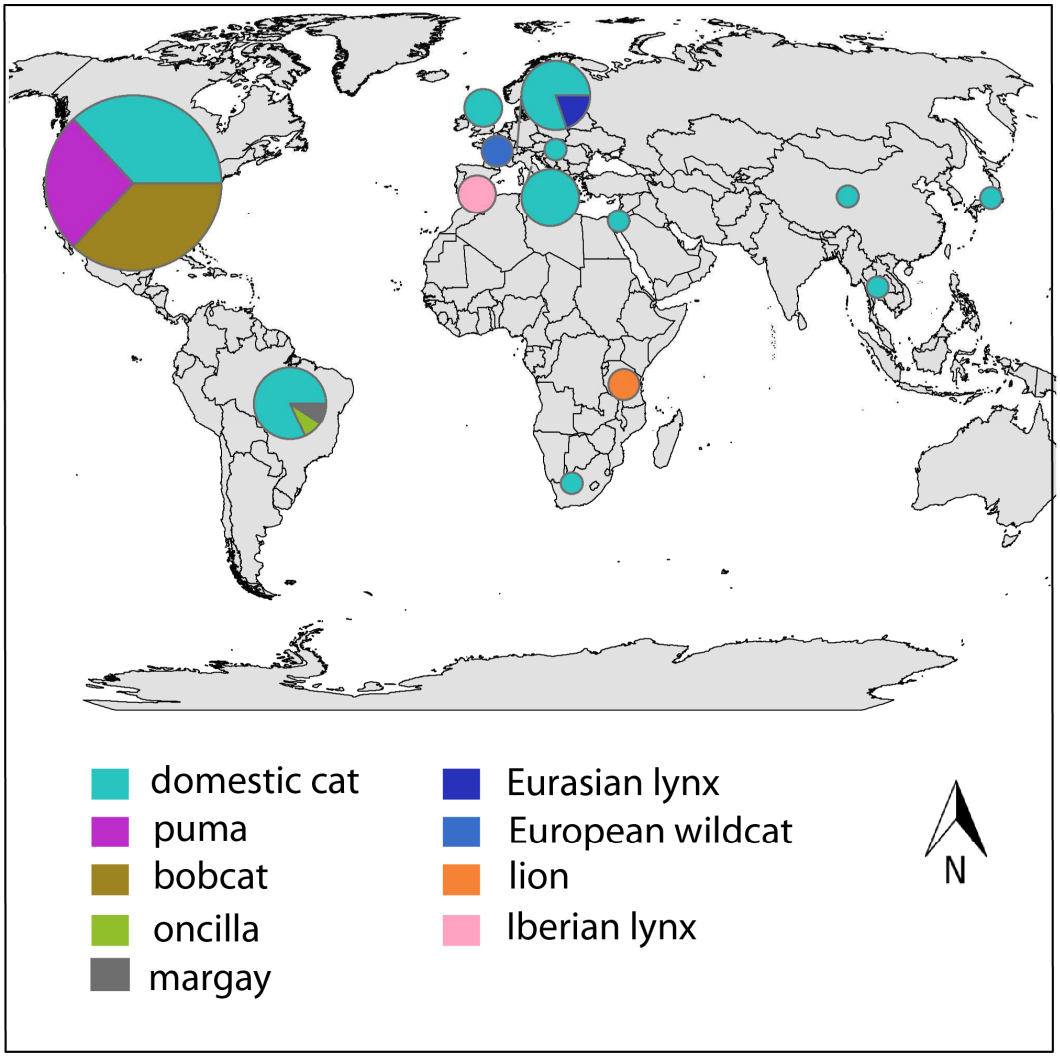
X1299; X1300; X1301; X1302; X1303; X1509; X1510; X905; X1537*; X913R1*; X192*; X1513*; X1304*	13	*L. rufus*	USA	CA-NLA
X1065; X384; X599	3	*L. rufus*	USA	CA-SLA
X1054; X1064; X1076; X433R1; X1346*	5	*P. concolor*	USA	CO-FR
X1058; X1131; X403; X686*; X224	5	*P. concolor*	USA	CO-WS
X1582; X1591	2	*P. concolor*	USA	CA-NLA
X1207; X393; X704; X871; X952R1	5	*P. concolor*	USA	CA-SLA
Total	**60**			

Previously Described Samples

GenBank Accession No.	**No. Samples**	***Species***	**Origin**	
AM745338	1	*F. catus*	China	.
AY150974	1	*F. catus*	Israel	
AY150979	1	*F. catus*	South Africa	
AY150980; HE613254; AY150981	3	*F. catus*	UK	
AY529634	1	*F. catus*	Japan	
DQ157141; DQ157142; DQ157143; DQ157144; DQ157145; DQ157146;DQ157147; DQ157148;	8	*F. catus*	Switzerland	
EU128752	1	*F. catus*	Hungary	
EU839979; EU839980; EU839981; EU839982; EU839983; EU839984; EU839985	7	*F. catus*	Italy	
FJ004275; KF743737; KF743738; KF743739; U88564	5	*F. catus*	USA	
KM275248; KM275249; KM275250; KM275251; KM275252; KM275253; KM275254; KM275255; KM275256	9	*F. catus*	Brazil	
EU285281	1	*F. catus*	Thailand	
DQ825442; DQ825443	2	*F. silvestris*	France	
DQ825444; DQ825445; DQ825446	3	*Linx. Pardinus*	Spain	
DQ825452; DQ825453	2	*Panhero leo*	Tanzania	
DQ825440	1	*L. wiedii*	Brazil	
DQ825439	1	*L. tigrinus*	Brazil	
DQ825456; DQ825457	2	*L. lynx*	Switzerland	

AF338269 **	1	*S. sciureus*
Total	**50**	

Figure 3. Geographic origins of all host species from which *M. haemominutum* isolates were used for phylogenetic analyses (Table 4). Samples include novel isolates from North American felids (n=60) as well as previously described sequences from GenBank (n=49). The size of the pie chart is scaled to the number of samples included from each region (largest = 65; smallest= 1).



Our final alignment of directly sequenced samples consisted of high-quality, unambiguous reads of ≥ 2x coverage, sequenced in both directions. We trimmed all sequences to equal length of 1238 nucleotides (including indels). Our final alignment (excluding the outgroup) included 159 variable positions and 103 informative characters (i.e. where alternative nucleotides were shared between two or more haplotypes).

PCR amplification

Genomic DNA was extracted from whole blood using QIAamp DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA). We amplified the 16S rRNA gene with two pairs of forward and reverse PCR primers used in previous studies (Barker 2011; Criado-Fornelio et al. 2003; Pitulle et al. 1999), with a single nucleotide modification (T to C) to the Pitulle et al. (1999) 8F universal primer. These primers amplified a total of 1484 nucleotides, with an overlap of 595 base pairs (Table 5).

PCR methodology was adapted from Criado-Fornelio et al. (2003) with the substitution of HotStarTaq DNA polymerase (Qiagen Inc.) in place of Amplitaq Gold DNA polymerase (Applied Biosystems, Inc., Foster City, CA, USA) for most samples. Reaction mixtures (25 µL) contained 12.5 µL HotStarTaq, 9 µL sterile-filtered PCR water, 0.5 µL of each forward and reverse primer, and 2.5 µL DNA. For some samples, the master mix was doubled from 25 to 50 µL; all components of reaction mixtures were likewise doubled for these samples. We used a GeneAmp PCR System 9700 PCR thermocycler (Applied Biosystems) for all amplifications. Having determined optimal primer annealing temperature using an annealing gradient, the final thermocycling profile was as follows: 94 °C for 10 min followed by 40 cycles of (95 °C for 30 sec; 52 °C for 30 sec; 72 °C for 60 sec), followed by 72 °C for 10 min. All PCR products as well as positive and negative controls

Table 5. Primers used for amplification of 16S rDNA. Nucleotide position numbers correspond to base pair positions within the fully sequenced genome of *M. haemominutum* (Barker et al. 2012), GenBank Accession No. HE613254. Each fragment was sequenced in both directions using each primer in turn to ensure that all nucleotides were called at least twice.

Primer	Direction	Sequence	Nucleotide Position	Amplified Fragment Length	Source
8F	Forward	5'-AGAGTTTGATCCTGGCTCAG-3' *	306,274	933	Pittule et al. 1999
908R	Reverse	5'-TGCTCCACCACTTGTTCA-3'	305,361	933	Criado-Fornelio et al. 2003
313F	Forward	5'-ATACGGCCCATATTCCTACG-3'	305,960	1268	Criado-Fornelio et al. 2003
1492R	Reverse	5'-GGTTACCTTACGACTT-3'	304,834	1268	Pitulle et al. 1999

* modified for our study from the original published version by a single nucleotide (T-C) in the 11th position (5'-3' direction)

were visualized under UV light using gel electrophoresis, using 1.5% agarose gel and EZ-Vision 6x DNA dye (Amresco, OH, USA).

Sequencing and alignment

PCR products were purified prior to sequencing, using either QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit (Qiagen Inc.), depending on the presence or absence of multiple bands, respectively. DNA concentrations varied widely among samples, yielding between 10 and 120 ng/μL.

Samples were directly sequenced in both directions in bulk at the University of Chicago Comprehensive Cancer Center; when fewer than 12 samples were sequenced at one time, we used the Proteomics and Metabolomics Facility at Colorado State

University. We accepted sequences with ≥ 2x high-quality coverage and upon visual examination using Geneious version 7.1.5 (http://www.geneious.com, Kearse et. al 2012). Additionally, we identified in GenBank another 49 previously described *M. haemominutum* sequences, as well as a *Candidatus Mycoplasma kahanei* sequence (host: *S. sciureus*) for use as an outgroup in our phylogenetic analysis (Table 4). We aligned all sequences using MAFFT version 7 method Q-INS-i (Katoh and Toh 2008) and trimmed all sequences at the 5' and 3' ends such that all sequences were the same length (1238 bp). We aligned each sequence to the 16S rRNA sequence extracted from the noncontiguous finished genome sequence of *Ca. Mycoplasma haemominutum* (GenBank accession no. HE613254).

Coinfections

We discovered a high rate of multiply-infected samples by closely inspecting what appeared to be low quality reads containing long sequences with double- and triple-peaked chromatograms. Upon examination, these chromatograms were determined to have arisen from co-infected individuals that carried two or more sequences that differed from each other because of insertion-deletion (indel) mutations. We compared alignments of sequence data from singly-infected individuals against the putative co-infected samples, and found that in most cases, frameshifts were clear and their positions corresponded with loci at which indels occurred in other sequences. Upon inspecting the sequences manually, we discovered that most putatively co-infected sequences with indel mutations realigned with known sequences when accounting for missing nucleotides. Subsequently, all reads with > 1 peak at any given nucleotide locus were visually examined for frameshifts indicative of multiple *Mhm* infections.

We then automated and streamlined the process used to identify the individual haplotypes in co-infected sequences using 1) Mixed Sequence Reader (Chang et al. 2012) to extract two distinct sequences from double-peaked chromatograms, 2) Geneious 7.1.5 (Kearse et al. 2012) to visually verify all heterozygous base calls, 3) SeqPHASE (Flot 2010) to format our data, and finally 4) the program PHASE (Stephens and Donnelly 2003) to reconstruct co-infected haplotypes. In PHASE, we ran 10,000 iterations with a thinning interval of 5 and a burn-in of 100. We used the original mutation model without recombination or stepwise mutation for multi-allelic loci (Stephens et al. 2001).

Phylogenetics

We used the BEAUTi graphical user interface for program BEAST to input parameters for a Bayesian Markov chain Monte Carlo (MCMC) analysis of 1.2E8 iterations (Drummond and Rambaut 2007; Drummond et al. 2012). Using jModelTest2 (Darriba et al. 2012), we used AIC criteria to select the Generalized Time Reversible model with both invariable sites and gamma-distributed rate variation among sites (GTR + I + G). We discarded the first 10% of iterations as burn-in, and recorded parameters every 12000 trees. We used default priors and assumed a coalescent model with a constant population size of *Mhm* over time. Nodes of the tree were estimated using substitutions per site, with probabilities derived from the posterior distribution. We accepted the resulting Maximum Clade Credibility Tree (MCC) upon ensuring convergence of parameter estimates and effective sample sizes (ESS) of > 200, where the ESS represents a measurement of adequate mixing of parameters and a lack of auto-correlation between states. The MCC represents a model-averaged tree in which the contribution of each sampled tree to the final model is proportional to its posterior likelihood. We used the program Tracer 1.6 (Rambaut and

Drummond 2013) to calculate all marginal posterior probabilities and point estimates from the posterior distribution. We superimposed all tree elements onto our MCC tree using FigTree v1.4.2 (Rambaut 2007).

Reconstruction of global cross-species transmission events

We partitioned our Bayesian phylogenetic analysis to 1) test whether the host or geographic origin of a *Mhm* sample better explained the evolutionary relationship between *Mhm* isolates; 2) estimate mean pairwise transition rates between all host-to-host and location-to-location combinations; and 3) identify the most likely ancestral host at each node along our MCC tree. Following protocol from previous studies (Faria et al. 2013; Hayman et al. 2013; Lemey et al. 2009; Streicker et al. 2010) we assigned discrete traits to each *Mhm* genotype based on host species ("host") and geographic origin ("location"). For analyses of host species and geographic origin, we established a prior of a diffuse gamma distribution with shape and scale parameters set to one and an initial value of one. This prior represents a mean estimate of a single state-to-state transition over all trees. We allowed for the possibility of assymetrical directionality in pairwise combinations, meaning, for example, that a transition of host species from *F. catus* to *L. rufus* need not occur with the same frequency as *L. rufus* to *F. catus*. We then conducted a posterior simulation-based analog of Akaike's Information Criteria through Markov chain Monte Carlo (AICM) analysis (Baele et al. 2012; Raftery et al. 2007) to ascertain whether models based on host species or geographic origin best described our data as evolutionary drivers of relationships between *Mhm* genotypes.

Host-to-host transition rates were estimated from the means of the posterior distributions over all sampled trees. We inspected these means and intervals of highest

probability density (HPD) to assess transitional trends with respect to the established prior (i.e. whether the transition rate was greater or less than 1, the mean of the gamma distribution).

The ancestral host assigned to each node is represented as a state probability (SP) derived from the posterior distribution (Figure 6). When both the posterior probability (PP) and the state probability (SP) of a node were high (>0.95), we concluded strong support for the hypothesis that the host depicted at a particular node was the ancestral host at that particular branch in the tree.

RESULTS

Modeling of transmission mechanisms

We sampled and tested 716 felids (157 pumas, 257 bobcats, 302 domestic cats) for *Mhm,* determining that infection prevalence was highest in pumas (range: 0.33 – 0.76) and bobcats (range: 0.32 – 0.58) and generally lower in domestic cats (range: 0.06 – 0.32) across our study sites. There was no obvious difference in infection prevalence across study sites, except for NLA domestic cats having a higher prevalence relative to FR domestic cats (Table 1; Figure 2).

We modeled combinations of intra- and interspecific mechanisms of *Mhm* transmission and assessed the likelihood of these models relative to observed prevalence across felids and study sites (Appendix 1). Each of the nine top models ($\Delta AIC < 2$) supported aggressive encounters as a mechanism of intra-specific transmission, and vectors as a mechanism of both intra- and interspecific transmission (Table 3). The top four models supported predation as a mechanism of interspecific transmission. We estimated infection prevalence as the model average across the entire model set (Appendix 1) and found a strong correlation between predicted and observed infection prevalence (slope = 0.77, intercept 0.09; spearman correlation = 0.902) (Figure 4). Using random forest analysis to assess importance of hypothesized transmission mechanisms, we observed strong support for aggressive encounters as a primary mechanism of intra-specific transmission (1b, Figure 5). Mechanisms of inter-specific transmission were less distinct, possibly indicating a relative rarity of these events. Aggressive encounters of bobcat and puma with domestic cats (2b, as a mechanism of interspecific transmission) and a

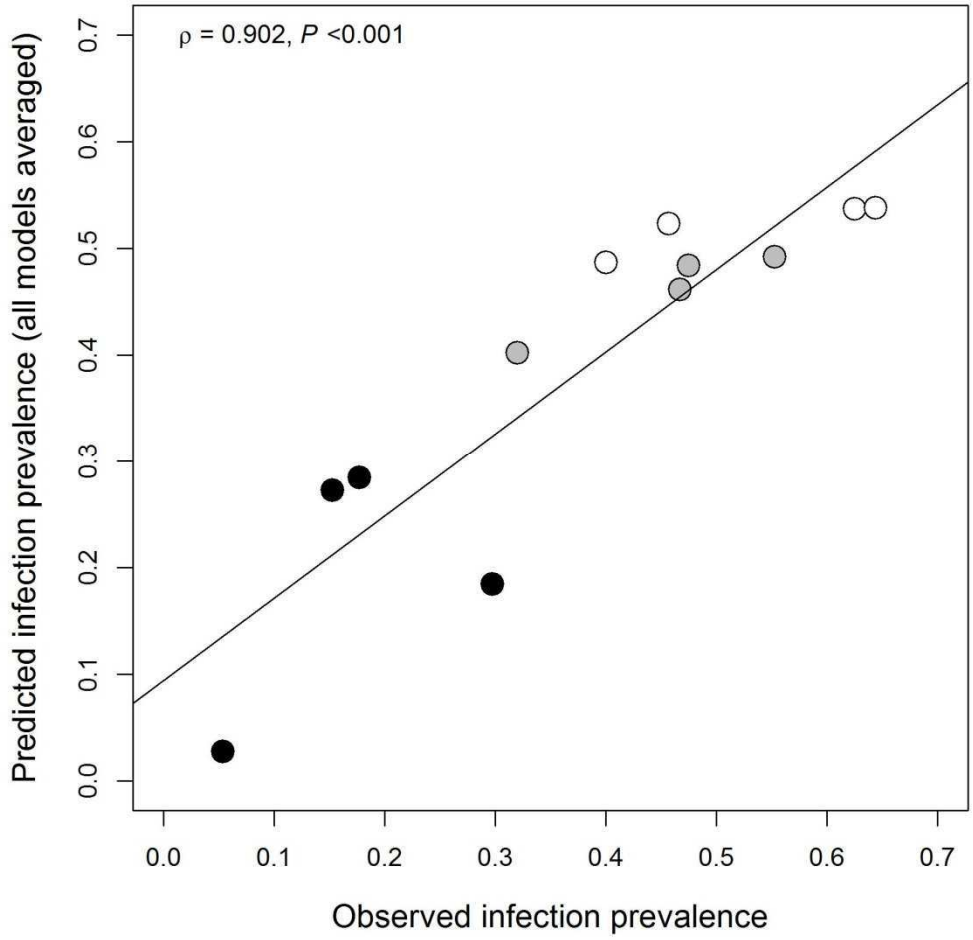


Figure 4. Relationship between observed '*Ca.* M. haemominutum' prevalence of infection (I/N) to prevalence of infection as estimated by all models/*a priori* hypotheses averaged. Closed, open and grey circles represent domestic cat, bobcat and puma respectively. Spearman correlation $\rho = 0.902$ $P < 0.001$.

generalist vector (3a, as a mechanism of intra- and interspecific transmission) were the next most supported models of transmission (Figure 5).

Phylogenetics and cross-species transmission

Analysis of *Mhm* tree topology revealed clustering patterns consistent with higher levels of intraspecific than interspecific transmission (Figure 6).

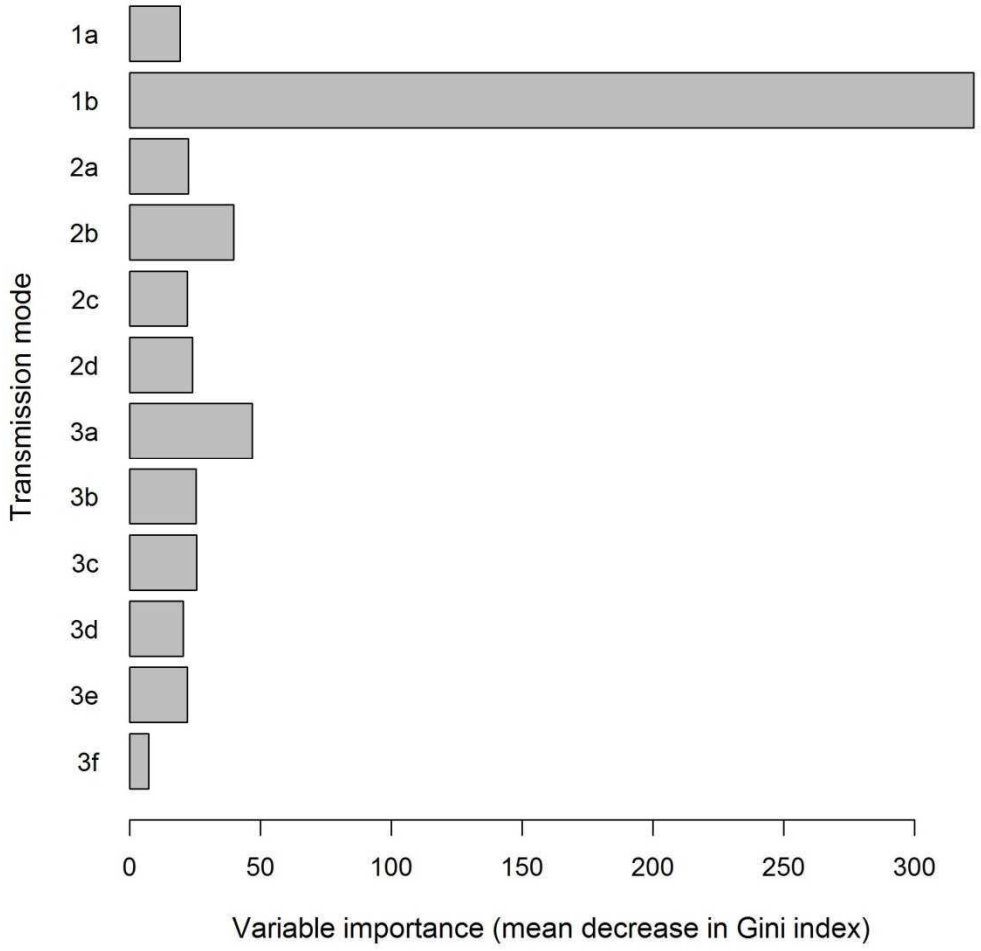
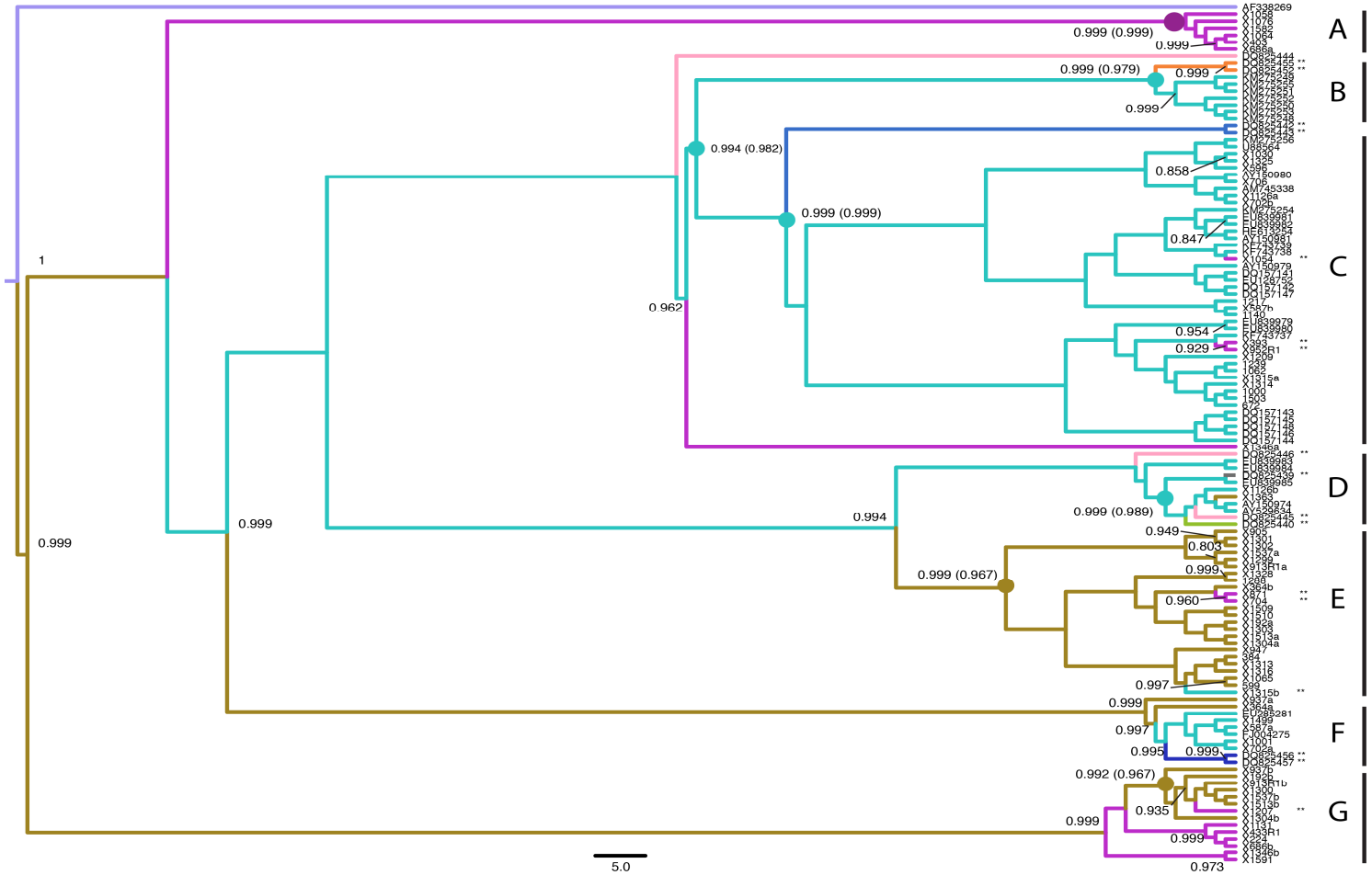


Figure 5. Variable importance plot comparing relative importance of hypothesized transmission modes (Table 2) across all models. Variable importance calculated from random forests classification, demonstrating the relative importance of transmission modes (mean decrease in Gini index) for *Mhm* transmission, as measured by model selection (ΔAIC).

With a few exceptions, *Mhm* lineages largely assort according to host species with pumas represented in clades A and G, bobcats in clades E, F, and G, and domestic cats in clades B, C, D, and F. For all other wild felids, our sample size was insufficient to detect species-specific clades. However, some long branches (i.e. F. silvestris (Accession Nos.



Host (branches)

domestic cat
bobcat
puma
lion
margay
oncilla
Eurasian lynx
Iberian lynx
European wildcat
outgroup (C.M kahanei)

Figure 6. Bayesian phylogeny and ancestral host state reconstruction of *Mycoplasma haemominutum* genotypes. Node labels without parentheses indicate posterior probabilities (PP). Node labels in parentheses indicate posterior host state probabilities (SP). PP values > 0.80 and SP values > 0.95 were indicated. We did not denote PP for nodes depicted in very recent branching events. For example, in clade A, all branching events carried a PP of 1, but we omitted this information on account of space constraints. Circles indicate nodes with both high PP and SP probabilities (>0.95), indicating strong support for estimated ancestral host species. Circle coloration corresponds to host species. Clades are assigned letters A-G for reference.

DQ825442 and DQ825443)) may represent a species-specific clade that would be revealed with greater sampling. Clades A and G depict sequences consisting exclusively of North American wild cats, with clade A representing divergent, puma-specific isolates, and clade G a mix of both puma- and bobcat-derived samples with clear divergence between isolates extracted from these two hosts. Isolates from pumas, bobcats, and domestic cats were all paraphyletic, possibly representing some frequency of cross-species transmission and/or host-switching in both more recent and deeper time (see Discussion).

Statistical support for particular nodes in the tree, as measured by posterior probability (PP), was mixed, with both recent splits and older clades supported. We could not distinguish any association between North American capture locations and similarity of *Mhm* isolates in either wild or domestic cat samples. At the global level, however, some clades show clustering of sequences from similar geographic origins (Figure 7). For example, in clade B, all domestic cat genotypes were sampled from Brazil, and clade C, domestic cat-derived genotypes were sampled from Switzerland. In general, however, host species informs tree topology more than sample origin, as is supported empirically by our AICM analysis (see below).

Exceptions to congruency between *Mhm* genotypes and the felid family tree occurred in all clades but one (Figure 6, clade A).

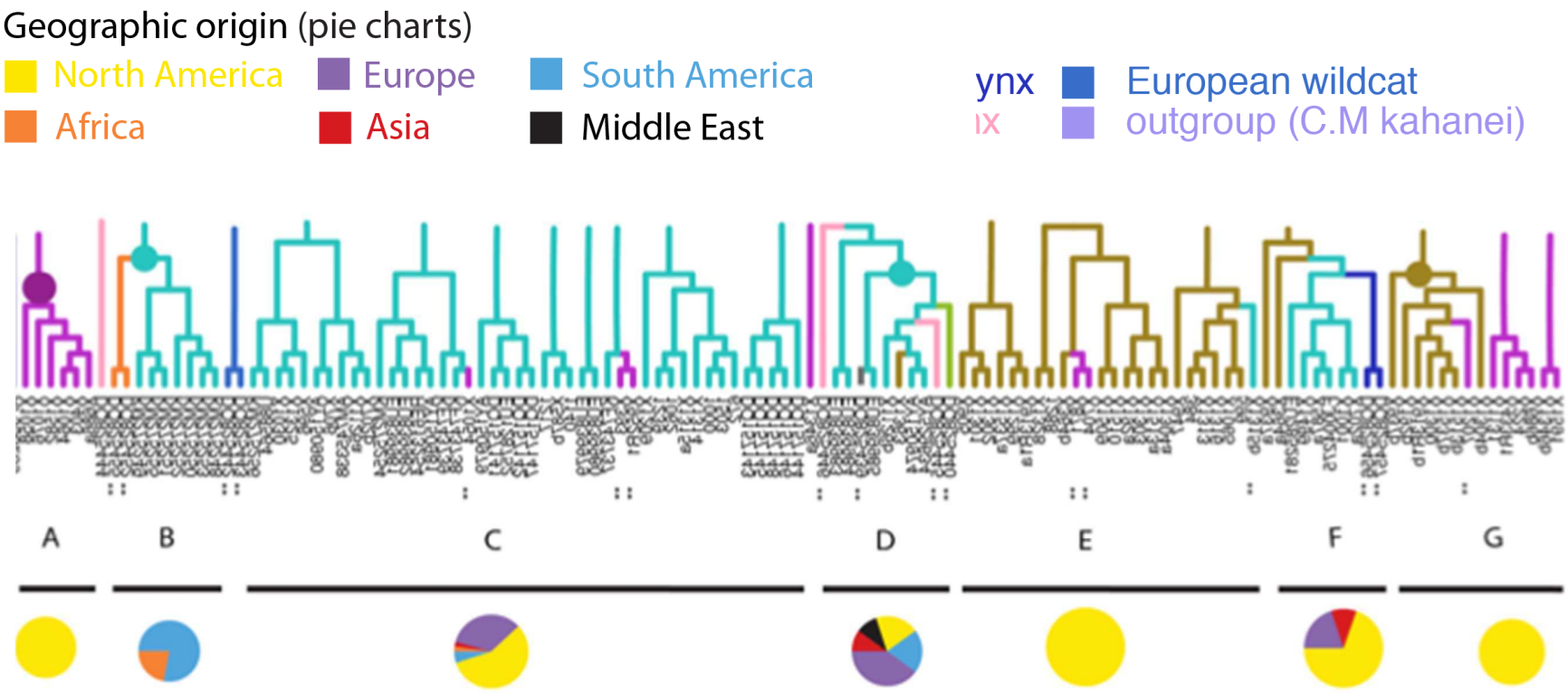


Figure 7. Phylogeny branch tips, labels, and clade assignments rotated 90 degrees clockwise (see Figure 6). Pie charts reflect the geographic origins of the sampled hosts within each *M. haemominutum* clade (A-G).

This pattern is consistent with infrequent cross-species transmission, primarily from lower to higher trophic levels. Our finer-scale North American data shows high sequence identity, or cross-species transmission, between three genotypes from pumas and the domestic cat genotypes within which they are nested (Figure 6, clade C). Similarly, two genotypes from puma showed high sequence identity to bobcat-derived isolates within the bobcat-dominant clade (Figure 6, clade E). In other cases, divergent *Mhm* genotypes from wild hosts clustered with domestic cat-derived isolates, suggesting less recent but still unidirectional cross-species transmission (Figure 6, clades B-G). In one instance, a genotype derived from a domestic cat clustered with genotypes from bobcats (Figure 6, clade E).

Reconstruction of global cross-species transmission events

AICM analyses revealed that the felid host from which *Mhm* samples originated (AICM=267.2) better explained the evolutionary relationships between *Mhm* genotypes than either the geographic origins of samples (AICM=454.2) or a null model (AICM=8,616.1).

Host-to-host transition rates reflect point estimates compiled from the means of the posterior distributions over all sampled trees. Of the 12 host-to-host transitions with a mean rate > 1, eight were transitions from domestic to wild species. Three were transitions from bobcat to puma, domestic cat, and Iberian lynx, though the latter was only slightly above 1 and therefore possibly indistinct from a random expectation. The remaining transition rate > 1 was from puma to bobcat.

Phylogenetic analyses provide strong posterior support (both PP and SP) across four major nodes for which the domestic cat (*F. catus) w*as determined to be the original host of *Mhm* (Figure 6, clades B, C, D). Notably, these clades tended to include *Mhm* isolates from

disparate hosts and geographic locations, indicating diversification and dispersal of *Mhm* on a global scale. For example, clade B includes a genotype primarily found in Brazilian domestic cats also found in a Tanzanian lion host; clade C includes a potential host-switch from domestic cat to European wild cat, and multiple North American cross-species transmissions from domestic cats to pumas. Clade D is the most diverse in terms of both geographic regions and species represented, with five of six sampled regions shown alongside four host-shifts, from domestic cat to Iberian lynx, bobcat, oncilla and margay (Figure 6).

In contrast, *Mhm* clades with puma or bobcat host origins show minimal geographic dispersal, though cross-species transmissions still occur. In particular, clade E depicts multiple host-shifts over time between domestic cats and bobcats (though these were not strongly supported), as well as a well-supported pattern consistent with cross-species transmission from bobcat to puma. The topology of clade G likewise depicts multiple host shifts from bobcat to puma, with one well-supported host-shift from bobcat to puma.

DISCUSSION

Prevalence

Prevalence of *Mhm* varied widely among populations, species, and geographic areas, in this study and others (Barker and Tasker 2013; Willi et al. 2007). Prevalence of *Mhm* in bobcats and pumas in our study sites was generally higher than domestic cats and fell within the range of *Mhm* prevalence reported in previous studies of wild cats (0.10 – 0.96, Willi et al. 2007). Willi et al. (2007) further differentiated between free-ranging and captive individuals, noting that the prevalence of *Mhm* in free-ranging animals (0.54) was much higher than in zoo-born (0.05) or wild-caught (0.16) captive animals. Our puma and bobcat samples were derived from free-ranging cats, and are comparable to the overall prevalence reported in free-ranging wild cats (Willi et al. 2007).

Likewise, our prevalence rates for domestic cats were similar to those found in previous studies (0.08 – 0.47, see Barker and Tasker 2013), with the exception of the WS group, where prevalence was slightly lower (0.06). Our 0.035 coinfection rate of *Mhm* with other haemoplasma species (i.e. *M. haemofelis* or *M. turicensis*) was also similar to previous studies (range 0.001 – 0.13, mean 0.028; see Barker and Tasker 2013). However, we also found a high rate of coinfection of multiple *Mhm* genotypes, with > 0.40 (34/82) of all samples co-infected. This estimate is likely low, as we suspect many of the samples that failed to neatly sequence were multiply-infected.

Modeling of transmission mechanisms

To effectively manage disease threats to wild populations, an understanding of transmission dynamics is essential. In emerging and newly recognized pathogens, however,

such mechanisms are often unknown. The natural mechanism of haemoplasma transmission has not been identified, and recent studies cite this objective as important in haemoplasma research (Barker and Tasker 2013). The results of our AIC and random forest analysis strongly support recent studies implicating aggressive encounters as the primary mechanism of intra-specific disease transfer of *Mhm*, and predation as the logical extension for interspecific transmission (Dean et al. 2008; Museux et al. 2009; Woods et al. 2006). Our model results, however, also support generalist vectors as an ancillary mode of transmission.

That our modeling of interspecific transmission yielded greater uncertainty compared to intra-specific transmission may reflect the rarity of cross-species transmission. This conclusion is supported by our phylogenetic analysis, in which host-specificity is maintained in the overall topology of the *Mhm* tree, with cross-species transmission events occurring less frequently than divergence of *Mhm* within felid host species. Further, our phylogenetic analysis corroborates the support from our transmission models for predation as the primary mechanism of cross-species transmission. First, several genotypes sampled from wild felids share high sequence identity with domestic-derived isolates, and cluster within domestic-dominant clades; second, we see strong support for domestic cat genotypes as ancestral to clades in which wild cat genotypes occur; and third, genotypes from pumas comprise a divergent and species-specific clade. This latter topological pattern suggests the existence of an ancient genotype that may not have ascended to a higher trophic level, because among the North American felids sampled pumas are the apex predator.

These results, in combination with laboratory experiments by Woods (2006), Dean (2008) and Museux (2009), support direct transmission as the primary mechanism of *Mhm* infection. The success of our modeling approach, as indicated by phylogenetic support and a strong correlation between observed and predicted prevalence (Figure 4), demonstrates the utility of this method in identifying transmission mechanisms in other pathogen systems. These results also enable us to glean information about the trophic and aggressive behaviors of ecologically pivotal yet secretive felids, which are notoriously difficult to study directly.

Phylogenetics of Mhm

Mhm genotypes in pumas, bobcats, and domestic cats are paraphyletic, indicating the existence of multiple divergent *Mhm* strains as well as multiple host shifts in both deeper and recent time. The emergent phylogenetic pattern of puma and bobcat *Mhm* genotypes is similar to that of FIV (Franklin et al. 2007) and GHV (Troyer et al. 2014), viral infections of North American felids also transmitted through blood-to-blood contact. In recent phylogenetic analyses of these pathogens, multiple major genotypes of each virus exist, of which one is specific to pumas and another is shared between pumas and bobcats. For example, Franklin (2007) describes FIV/PcoB as a puma-specific strain, but FIV/PcoA as a strain infecting both pumas and bobcats, the result of a possible host jump from bobcat to puma. Similarly, Troyer et al. (2014) describes PcoGHV1 as a genotype infecting pumas only, and LruGHV1 as infecting both pumas and bobcats, for which bobcats are the presumed primary host.

Our results corroborate this pattern, as our phylogeny depicts a clear, divergent, puma-specific clade as well as multiple clades in which bobcats and pumas are intermixed.

Furthermore, we see strong directional support for bobcat-to-puma transmission in two instances based on our Bayesian analysis of ancestral states as well as a high bobcat-to-puma host state transition rate (Figure 6, clades E and G; Table 6).

Table 6. Posterior estimates of state-to-state transition rates based on host species of *Mhm*, from the partitioned Bayesian phylogenetic analysis of *M. haemominutum*. Only transition rates with a mean estimate > 1 are listed.

Host Transitions	Mean Transition Rate	95% HPD Interval
F. catus -> F. silvestris	1.5667	[0.0549, 3.7809]
F. catus -> L. lynx	1.1676	[9.023E-4, 3.067]
F. catus -> L. lynx	2.2204	[0.1432, 4.7186]
F. catus -> L. rufus	2.4453	[0.1008, 5.3581]
F. catus -> L. tigrinus	1.2943	[8.8518E-4, 3.2097]
F. catus -> L. wiedii	1.2927	[5.8391E-3, 3.1862]
F. catus -> P. concolor	2.583	[0.2266, 5.3961]
F. catus -> P. leo	1.3586	[0.0153, 3.2302]
L. rufus -> F. catus	2.5413	[0.0199, 5.4965]
L. rufus -> L. pardinus	1.0043	[7.5016E-5, 2.8844]
L. rufus -> P. concolor	3.073	[0.4331, 6.4593]
P. concolor -> L. rufus	1.2063	[1.1911E-4, 3.3216]

HPD = Highest Probability Density

The *L. rufus* to *P. concolor* transition rate was the highest of all estimated rates (3.073), possibly reflecting a long period of co-existence and interaction between these two species. Interestingly, our results demonstrate likely bobcat-to-puma transmissions in two different time periods. Clade G depicts host-switching in deeper time in which an interspecific transmission event likely led to within-species propagation and diversification. Ultimately, puma-specific and bobcat-specific monophyletic clades emerged from this host-switching event, though a subsequent well-supported bobcat-to-puma transmission is depicted within the bobcat cluster. Clade E depicts two cross-species transmission events in more recent time, in which two pumas share sequences nearly identical to contemporary bobcat sequences within the monophyletic bobcat host clade.

The similarity between phylogenetic patterns of FIV, GHV, and *Mhm* provides further support both for host-switching in feline pathogens via predation events, and also for blood-to-blood contact as a transmission mechanism of *Mhm.*

Reconstruction of global cross-species transmissions

Domestic cats occur on the edges of urbanized areas and can serve as a reservoir for many pathogens that can infect wild cats (Brown et al. 2008; Carver 2016; Paul-Murphy et al. 1994; Riley et al. 2004; Roelke et al. 1993a). Many previous studies have identified nondomestic felid viral and bacterial strains with high sequence identity to domestic strains (Brown et al. 2008; Chomel et al. 2016; Franklin et al. 2007; Lagana et al. 2013; Troyer et al. 2014) but phylogenetic analyses alone do not provide definitive evidence of directionality of transmission. Our ancestral state reconstruction represents a new method for assessing directionality in felid disease transfer, though the method has been used in studies of Influenza-A in humans, rabies in dogs and bats, and *Bartonella* spp. in bats and rats (Lemey et al. 2009, Streicker 2010, Hayman 2013, McKee 2016).

We determined empirically using AICM analysis what was qualitatively evident from our phylogeny: host species informs the structure of our tree more than the geographic origin of the isolate. This conclusion supports direct transmission as the primary mechanism of pathogen transmission both within host species and between different felids. Alternatively, we would expect the opposite topological pattern and AICM results if vector-borne transmission was common. In such a scenario, we would expect generalist vectors to circulate similar sequences within geographic areas, which would dilute or negate the underlying pattern of host-specificity because of relaxed barriers to cross-species transmission.

Our results are striking, and highly supportive of the hypothesis that domestic cats are a source of *Mhm* infection on a global scale. Eight of 12 transitions with a mean rate > 1 were transferred from domestic to non-domestic host species. Of the remaining four transitions, three are ecologically relevant. Two are transitions from bobcats to the alternative hosts puma and domestic cat, both of which are depicted in our MCC tree and likely to occur in natural settings. The estimated rate for the puma-to-bobcat transition is much lower than its reverse, a result expected from the propensity of pumas to killing and/or consuming bobcats (Cashman et al. 1992; Harveson et al. 2000; Hass 2009; Koehler and Hornocker 1991). It is not entirely implausible, however, that a bobcat could survive an aggressive encounter with a puma and perpetuate an acquired infection. Similarly, it is not implausible that a domestic cat could survive an aggressive encounter with a bobcat. The remaining host state transition with a mean >1 is from bobcat to Iberian lynx, an ecologically improbable scenario given the lack of overlap between these species' ranges. This result may be random, given the estimate is only slightly above 1 and not highly distinct from the prior, or possibly an artifact of greater sampling effort of bobcats relative to other nondomestic cats. Future studies could resolve this issue by genotyping more isolates from these and other host species. Though our methodology identifies bobcats as the ancestral host of all *Mhm* genotypes in our phylogeny, this result has low posterior support and is almost certainly a sampling artifact. Wild felids outside North America are not sufficiently represented in this analysis to unambiguously identify the ancestral host of all *Mhm*.

The domestic cat was strongly supported as the ancestral host of at least 4 major contemporary clades of *Mhm,* each with broad geographic and host species distribution. In

contrast, clades for which pumas or bobcats were found to be ancestral hosts show minimal geographic dispersal of *Mhm*. This result suggests that as domestic cats accompany human companions into the broad reaches of the globe, they are concomitantly responsible for the spread of haemoplasmosis into their rarer, wild relatives.

Though some studies have shown that the pathogenicity of haemoplasmas increases with comorbid retroviral infection (Luria et al. 2004), *Mhm* is widely considered apathogenic, and poses little risk to felids on its own. *Mhm* may represent a worst-case transmission scenario, however, in that it occurs with high prevalence on a global scale, may be transmitted both directly contact and via generalist vectors, does not attenuate the life span or contact patterns of its host, and appears to infect alternative hosts with relative ease. The striking pattern of global dispersal via transmission from domestic cats indicates *Mhm* may be sentinel with regards to directly transmitted feline pathogens with relaxed barriers to cross-species transmission. Wild felids are already experiencing stressors associated with habitat degradation (Schipper et al. 2008; Wilcove et al. 1998), range restriction and fragmentation (Crooks 2002; Crooks et al. 2011; Gaona et al. 1998), poaching (Kenney et al. 1995), persecution (Inskip and Zimmermann 2009), prey depletion (Karanth and Stith 1999), climate change (Parmesan and Yohe 2003), and demographic reductions that may result in inbreeding depression (O'Brien and Yuhki 1999; Roelke et al. 1993b). Under these compromised circumstances, the introduction of infectious disease may become increasingly common and increasingly catastrophic.

CONCLUSION

Management options are, however, available, as Cleaveland (2007) and Meli (2009) discussed subsequent to the devastating CDV and FeLV outbreaks in African lions and Iberian lynxes, respectively. Because tools for managing disease in wildlife are relatively limited, disease may be controlled within domestic reservoirs via vaccination, treatment, physical separation from the wild species if possible, or population control via spay/neuter campaigns or demographic reduction (Cleaveland 2007; Meli 2009). Vulnerable wild populations may benefit from increased surveillance (Cleaveland et al. 2007), and vaccination campaigns have been used in wildlife with success in Florida panthers (Cunningham et al. 2008), Iberian lynx (López et al. 2009), and Island foxes (Cleaveland et al. 2006; Timm et al. 2000).

Ecological researchers may wish to explore *Mhm* as a possible model system for felid transmission dynamics via whole genome sequencing, given the small size of its genome and high prevalence in cats worldwide. Researchers of microbiology or pathology may be interested in patterns of selection in virulence genes, genomic rearrangements that contribute to bacterial phenotype, and the effects of co-infection on *Mhm* evolution. More extensive sampling of wild cats outside North America may also elucidate the evolutionary origins of the feline haemoplasmas, and provide further resolution of transmission pathways. Our research contributes to the growing body of literature on disease transmission among felids, and provides relatively novel investigative methodologies through which researchers can model transmission mechanisms in emerging pathogens, and use genetic data to corroborate their results.

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APPENDIX

A-1. *A priori* hypotheses/models of intra- and inter-specific *M. haemominutum* transmission (I/N), where the number of infecteds (I) for each hypothesis is represented by the following species-specific combination of expressions. See Table 2 for expressions, categories, and parameter definitions. Model fit based on Akieke Information Criteria (AIC).

A priori hypothesized transmission (Model #)	I_D	I_B	I_P	K	-2LOG(L)	AIC	ΔAIC	Weight (%)
Social contact								
1a	$1a_1$	$1a_2$	$1a_3$	3	86.85	92.85	7.88	0.267
Aggressive encounters								
2a	$1b_1$	$1b_2$	$1b_3$	3	93.77	99.77	14.80	0.008
Social contact + Aggressive encounters								
3a	$1a_1+1b_1$	$1a_2+1b_2$	$1a_3+1b_3$	6	77.50	89.50	4.53	1.425
Social contact + Predation								
4a	$1a_1-2a_1$	$1a_2$	$1a_3+2a_1$	4	88.57	96.57	11.60	0.042
4b	$1a_1-2b_1-2b_2$	$1a_2+2b_2$	$1a_3+2b_1$	5	85.56	95.56	10.60	0.069
4c	$1a_1-2c_1$	$1a_2-2c_3$	$1a_3+2c_1+2c_3$	5	84.22	94.22	9.25	0.135
4d	$1a_1-2d_1-2d_2$	$1a_2+2d_2-2d_3$	$1a_3+2d_1+2d_3$	6	81.51	93.51	8.54	0.192
Social contact + Aggressive encounters + Predation								
5a	$1a_1+1b_1-2a_1$	$1a_2+1b_2$	$1a_3+1b_3+2a_1$	7	76.92	90.92	5.95	0.701
5b	$1a_1+1b_1-2b_1-2b_2$	$1a_2+1b_2+2b_2$	$1a_3+1b_3+2b_1$	8	74.42	90.42	5.45	0.901
5c	$1a_1+1b_1-2c_1$	$1a_2+1b_2-2c_3$	$1a_3+1b_3+2c_1+2c_3$	8	73.57	89.57	4.60	1.376
5d	$1a_1+1b_1-2d_1-2d_2$	$1a_2+1b_2+2d_2-2d_3$	$1a_3+1b_3+2d_1+2d_3$	8	74.67	90.67	5.70	0.795

Vector-borne								
6a	$3a_1$	$3a_2$	$3a_3$	1	88.00	90.00	5.03	1.111
6b	$3b_1$	$3b_2$	$3b_3$	3	86.90	92.90	7.93	0.261
6c	$3c_1$	$3c_2$	$3c_3$	2	91.66	95.66	10.69	0.066
6d	$3d_1$	$3d_2$	$3d_3$	3	85.94	91.94	6.98	0.420
6e	$3e_1$	$3e_2$	$3e_3$	2	87.54	91.54	6.57	0.513
Vector-borne + Aggressive encounters								
7a	$3a_1+1b_1$	$3a_2+1b_2$	$3a_3+1b_3$	4	78.86	86.86	1.89	5.343
7b	$3b_1+1b_1$	$3b_2+1b_2$	$3b_3+1b_3$	6	74.77	86.77	1.80	5.578
7c	$3c_1+1b_1$	$3c_2+1b_2$	$3c_3+1b_3$	4	78.86	86.86	1.89	5.343
7d	$3d_1+1b_1$	$3d_2+1b_2$	$3d_3+1b_3$	6	74.74	86.74	1.77	5.681
7e	$3e_1+1b_1$	$3e_2+1b_2$	$3e_3+1b_3$	5	78.67	88.67	3.70	2.159
Vector-borne + Predation								
8a1	$3a_1-2a_1$	$3a_2$	$3a_3+2a_1$	2	88.00	92.00	7.03	0.409
8b1	$3a_1-2b_1-2b_2$	$3a_2+2b_2$	$3a_3+2b_1$	3	86.12	92.12	7.15	0.385
8c1	$3a_1-2c_1$	$3a_2-2c_3$	$3a_3+2c_1+2c_3$	3	87.22	93.22	8.25	0.222
8d1	$3a_1-2d_1-2d_2$	$3a_2+2d_2-2d_3$	$3a_3+2d_1+2d_3$	4	86.01	94.01	9.04	0.150
8a2	$3b_1-2a_1$	$3b_2$	$3b_3+2a_1$	4	89.82	97.82	12.85	0.022
8b2	$3b_1-2b_1-2b_2$	$3b_2+2b_2$	$3b_3+2b_1$	5	86.00	96.00	11.03	0.055
8c2	$3b_1-2c_1$	$3b_2-2c_3$	$3b_3+2c_1+2c_3$	5	84.68	94.68	9.71	0.107
8d2	$3b_1-2d_1-2d_2$	$3b_2+2d_2-2d_3$	$3b_3+2d_1+2d_3$	6	81.47	93.47	8.50	0.196
8a3	$3c_1-2a_1$	$3c_2$	$3c_3+2a_1$	3	87.54	93.54	8.57	0.190
8b3	$3c_1-2b_1-2b_2$	$3c_2+2b_2$	$3c_3+2b_1$	4	85.77	93.77	8.80	0.169
8c3	$3c_1-2c_1$	$3c_2-2c_3$	$3c_3+2c_1+2c_3$	4	87.26	95.26	10.29	0.080
8d3	$3c_1-2d_1-2d_2$	$3c_2+2d_2-2d_3$	$3c_3+2d_1+2d_3$	5	85.79	95.79	10.82	0.061

8a4	$3d_1$-$2a_1$	$3d_2$	$3d_3$+$2a_1$	4	85.09	93.09	8.12	0.237
8b4	$3d_1$-$2b_1$-$2b_2$	$3d_2$+$2b_2$	$3d_3$+$2b_1$	5	84.00	94.00	9.03	0.150
8c4	$3d_1$-$2c_1$	$3d_2$-$2c_3$	$3d_3$+$2c_1$+$2c_3$	5	86.29	96.29	11.32	0.048
8d4	$3d_1$-$2d_1$-$2d_2$	$3d_2$+$2d_2$-$2d_3$	$3d_3$+$2d_1$+$2d_3$	6	85.20	97.20	12.23	0.030
8a5	$3e_1$-$2a_1$	$3e_2$	$3e_3$+$2a_1$	3	87.54	93.54	8.57	0.190
8b5	$3e_1$-$2b_1$-$2b_2$	$3e_2$+$2b_2$	$3e_3$+$2b_1$	4	87.06	95.06	10.09	0.088
8c5	$3e_1$-$2c_1$	$3e_2$-$2c_3$	$3e_3$+$2c_1$+$2c_3$	4	87.76	95.76	10.79	0.062
8d5	$3e_1$-$2d_1$-$2d_2$	$3e_2$+$2d_2$-$2d_3$	$3e_3$+$2d_1$+$2d_3$	5	85.78	95.78	10.81	0.062
Vector-borne + Aggressive encounters + Predation								
9a1	$3a_1$+$1b_1$-$2a_1$	$3a_2$+$1b_2$	$3a_3$+$1b_3$+$2a_1$	5	76.04	86.04	1.07	8.050
9b1	$3a_1$+$1b_1$-$2b_1$-$2b_2$	$3a_2$+$1b_2$+$2b_2$	$3a_3$+$1b_3$+$2b_1$	6	82.07	94.07	9.10	0.145
9c1	$3a_1$+$1b_1$-$2c_1$	$3a_2$+$1b_2$-$2c_3$	$3a_3$+$1b_3$+$2c_1$+$2c_3$	6	74.27	86.27	1.30	7.165
9d1	$3a_1$+$1b_1$-$2d_1$-$2d_2$	$3a_2$+$1b_2$+$2d_2$-$2d_3$	$3a_3$+$1b_3$+$2d_1$+$2d_3$	7	73.77	87.77	2.80	3.386
9a2	$3b_1$+$1b_1$-$2a_1$	$3b_2$+$1b_2$	$3b_3$+$1b_3$+$2a_1$	7	78.03	92.03	7.06	0.403
9b2	$3b_1$+$1b_1$-$2b_1$-$2b_2$	$3b_2$+$1b_2$+$2b_2$	$3b_3$+$1b_3$+$2b_1$	8	75.24	91.24	6.27	0.599
9c2	$3b_1$+$1b_1$-$2c_1$	$3b_2$+$1b_2$-$2c_3$	$3b_3$+$1b_3$+$2c_1$+$2c_3$	8	73.06	89.06	4.09	1.776
9d2	$3b_1$+$1b_1$-$2d_1$-$2d_2$	$3b_2$+$1b_2$+$2d_2$-$2d_3$	$3b_3$+$1b_3$+$2d_1$+$2d_3$	9	75.98	93.98	9.01	0.152
9a3	$3c_1$+$1b_1$-$2a_1$	$3c_2$+$1b_2$	$3c_3$+$1b_3$+$2a_1$	6	73.99	85.99	1.02	8.265
9b3	$3c_1$+$1b_1$-$2b_1$-$2b_2$	$3c_2$+$1b_2$+$2b_2$	$3c_3$+$1b_3$+$2b_1$	7	78.66	92.66	7.69	0.294
9c3	$3c_1$+$1b_1$-$2c_1$	$3c_2$+$1b_2$-$2c_3$	$3c_3$+$1b_3$+$2c_1$+$2c_3$	7	72.62	86.62	1.65	6.016
9d3	$3c_1$+$1b_1$-$2d_1$-$2d_2$	$3c_2$+$1b_2$+$2d_2$-$2d_3$	$3c_3$+$1b_3$+$2d_1$+$2d_3$	8	72.79	88.79	3.82	2.035
9a4	$3d_1$+$1b_1$-$2a_1$	$3d_2$+$1b_2$	$3d_3$+$1b_3$+$2a_1$	7	77.26	91.26	6.29	0.591

9b4	$3d_1+1b_1-2b_1-2b_2$	$3d_2+1b_2+2b_2$	$3d_3+1b_3+2b_1$	8	75.90	91.90	6.93	0.430
9c4	$3d_1+1b_1-2c_1$	$3d_2+1b_2-2c_3$	$3d_3+1b_3+2c_1+2c_3$	8	73.40	89.40	4.43	1.497
9d4	$3d_1+1b_1-2d_1-2d_2$	$3d_2+1b_2+2d_2-2d_3$	$3d_3+1b_3+2d_1+2d_3$	9	74.46	92.46	7.49	0.325
9a5	$3e_1+1b_1-2a_1$	$3e_2+1b_2$	$3e_3+1b_3+2a_1$	6	72.97	84.97	0.00	13.739
9b5	$3e_1+1b_1-2b_1-2b_2$	$3e_2+1b_2+2b_2$	$3e_3+1b_3+2b_1$	7	76.63	90.63	5.66	0.809
9c5	$3e_1+1b_1-2c_1$	$3e_2+1b_2-2c_3$	$3e_3+1b_3+2c_1+2c_3$	7	73.11	87.11	2.14	4.702
9d5	$3e_1+1b_1-2d_1-2d_2$	$3e_2+1b_2+2d_2-2d_3$	$3e_3+1b_3+2d_1+2d_3$	8	73.53	89.53	4.56	1.405
Environmental								
10a	$3f_1$	$3f_2$	$3f_3$	3	86.84	92.84	7.88	0.268
Environmental + Aggressive encounters								
11a	$3f_1+1b_1$	$3f_2+1b_2$	$3f_3+1b_3$	6	76.26	88.26	3.29	2.649