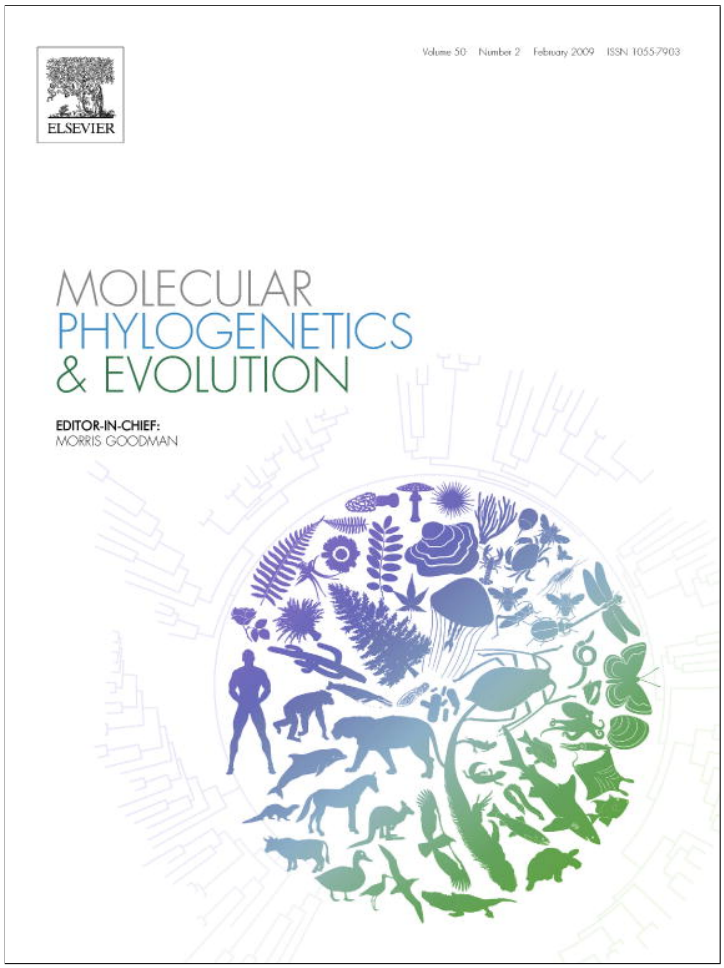

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Multigene analysis of phylogenetic relationships and divergence times of primate sucking lice (Phthiraptera: Anoplura)

Jessica E. Light *, David L. Reed

Florida Museum of Natural History, University of Florida, Dickinson Hall, P.O. Box 117800, Gainesville, FL 32611-7800, USA

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ABSTRACT

Cospeciation between hosts and parasites offers a unique opportunity to use information from parasites to infer events in host evolutionary history. Although lice (Insecta: Phthiraptera) are known to cospeciate with their hosts and have frequently served as important markers to infer host evolutionary history, most molecular studies are based on only one or two markers. Resulting phylogenies may, therefore, represent gene histories (rather than species histories), and analyses of multiple molecular markers are needed to increase confidence in the results of phylogenetic analyses. Herein, we phylogenetically examine nine molecular markers in primate sucking lice (Phthiraptera: Anoplura) and we use these markers to estimate divergence times among louse lineages. Individual and combined analyses of these nine markers are, for the most part, congruent, supporting relationships hypothesized in previous studies. Only one marker, the nuclear protein-coding gene Histone 3, has a significantly different tree topology compared to the other markers. The disparate evolutionary history of this marker, however, has no significant effect on topology or nodal support in the combined phylogenetic analyses. Therefore, phylogenetic results from the combined data set likely represent a solid hypothesis of species relationships. Additionally, we find that simultaneous use of multiple markers and calibration points provides the most reliable estimates of louse divergence times, in agreement with previous studies estimating divergences among species. Estimates of phylogenies and divergence times also allow us to verify the results of [Reed, D.L., Light, J.E., Allen, J.M., Kirchman, J.J., 2007. Pair of lice lost or parasites regained: the evolutionary history of anthropoid primate lice. BMC Biol. 5, 7.]; there was probable contact between gorilla and archaic hominids roughly 3 Ma resulting in a host switch of *Pthirus* lice from gorillas to archaic hominids. Thus, these results provide further evidence that data from cospeciating organisms can yield important information about the evolutionary history of their hosts.

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1. Introduction

A large portion of the world's biodiversity is comprised of parasitic organisms and because parasites are dependent upon their hosts for survival, understanding parasite evolutionary history can yield valuable information about host evolutionary history (Ashford, 2000; Whiteman and Parker, 2005; Hypsa, 2006; Nieberding and Olivieri, 2007; Reed et al., 2009). This is especially true if parasite speciation occurs as a result of, or at the same time as, host speciation (i.e., cospeciation). Parasites that have cospeciated with their hosts essentially track their hosts through time, therefore, they can serve as independent markers of host evolutionary history (Whiteman and Parker, 2005; Hypsa, 2006). Oftentimes, host data (e.g., molecular, morphological, or fossil data) are insufficient to resolve certain aspects of host history and in these cases data from a cospeciating parasite may provide vital information that can be used to infer events in host evolutionary history.

Sucking lice (Insecta: Phthiraptera: Anoplura) that parasitize primates are known to cospeciate with their hosts (Reed et al., 2004, 2007) and several recent studies have used this evolutionary trend as a starting point to infer novel aspects of primate history (Kittler et al., 2003, 2004; Reed et al., 2004, 2007; Raoult et al., 2008). For example, using louse molecular data, phylogenetic reconstructions, and estimates of divergence times, Reed et al. (2007) found that the louse species *Pthirus gorillae* and *Pthirus pubis* diverged approximately 3 million years ago (Ma). Given the host distribution of these two louse species (humans and gorillas, respectively) and because this divergence event was significantly more recent than the divergence of their hosts (Stauffer et al., 2001), Reed et al. (2007) inferred there was a host switch of *Pthirus* from gorillas to archaic hominids roughly 3 Ma. This host switch could have resulted from habitat sharing, predation, or other forms of contact between archaic hominids and gorillas. Thus, examination of parasite data can have broad implications for understanding host evolutionary history.

* Corresponding author. Fax: +1 352 846 0287.
E-mail address: jlight@flmnh.ufl.edu (J.E. Light).

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The conclusions of Reed et al. (2007) are dependent upon the results of their phylogenetic analyses, which were based on fragments of only two molecular markers. Use of only a few molecular markers to hypothesize species relationships is problematic because resulting trees may represent the evolutionary history of the gene rather than the species. Gene trees may differ from the species tree due to incomplete lineage sorting, gene duplication and extinction, hybridization, or lateral gene transfer (Maddison, 1997). These same processes also may cause individual gene trees to differ from one another. Although it has been assumed that analyses of multiple genes in a combined or concatenated framework will result in a strongly supported estimate of the species phylogeny (Chen and Li, 2001; Rokas et al., 2003; Driskell et al., 2004; Gadagkar et al., 2005), it is important to examine each locus individually to identify potential sources of topological incongruence. Combining information from genes with significantly different evolutionary histories could result in a lack of resolution, poor support, or incorrect species relationships in the resulting tree (Degnan and Rosenberg, 2006; Edwards et al., 2007; Kubatko and Degnan, 2007). In general, however, analyses of combined data sets potentially enable a more complete understanding of evolutionary relationships than analyses of single genes (Wiens, 2006; Ceotto et al., 2008). In addition to increasing confidence in phylogenetic relationships, analysis of multiple molecular markers can also be advantageous when estimating divergence times. Previous studies have found that analyses using multiple markers yield more precise estimates of divergence times compared to individual loci (Thorne and Kishino, 2002; Yoder and Yang, 2004; Noonan and Chippindale, 2006), especially when methods used to estimate divergences permit different evolutionary rates among markers (Thorne and Kishino, 2002; Drummond and Rambaut, 2007).

Previous louse studies have relied heavily on two molecular markers for phylogenetic analysis, the mitochondrial cytochrome *c* oxidase subunit 1 (COI) and the nuclear elongation factor 1 alpha (EF-1α) genes. These two genes are the most commonly used markers in louse systematics (Cruickshank et al., 2001; Johnson et al., 2001, 2003; Weckstein, 2004; Whiteman et al., 2004; Balakrishnan and Sorenson, 2007; Light and Hafner, 2007a,b; Stefka and Hypsa, 2008). Less often, other mitochondrial (Page et al., 2002; Kittler et al., 2003; Yoshizawa and Johnson, 2003; Smith et al., 2004; Hughes et al., 2007; Johnson et al., 2007; Whiteman et al., 2007) or nuclear markers (Johnson and Whiting, 2002; Barker et al., 2003; Kittler et al., 2003; Johnson et al., 2004; Leo and Barker, 2005; Light et al., 2008) have been employed in louse phylogenetic studies. Rarely, however, has there been a study with more than two independent loci (e.g., Johnson et al., 2007; Hughes et al., 2007). Therefore, even though lice have frequently served as important markers to infer host evolutionary history (e.g., Reed et al., 2004, 2007; Raoult et al., 2008), few molecular makers have been used (and are available) to resolve phylogenetic questions, estimate divergence times, or address evolutionary questions in lice.

Given the potential utility of parasites to infer the evolutionary history of their hosts as well as the many benefits of examining multiple molecular markers, the aim of the current study is to thoroughly examine a multigene data set in primate lice to test the relationships postulated in previous studies (Reed et al., 2004, 2007). Furthermore, we used this multigene data set to estimate divergence times among louse lineages to verify the results of Reed et al. (2007) postulating a host switch between humans and gorillas. The multigene data set examined in this study consists of several mitochondrial and nuclear protein-coding genes commonly used in insect systematics (Simon et al., 1994; Brower and DeSalle, 1998; Danforth et al., 2006; Wild and Maddison, 2008) but rarely used in lice. We therefore evaluate the phylogenetic utility of each marker in lice and potentially increase the number of loci currently available for phylogenetic analyses in parasites.

2. Materials and methods

2.1. Taxon sampling and data collection

We collected samples of lice belonging to the families Pediculidae (*Pediculus humanus* from humans and *Pediculus schaeffi* from chimpanzees), Pthiridae (*Pthirus gorillae* from gorillas and *Pthirus pubis* from humans), Pedicinidae (*Pedicinus badii* from red colobus monkeys), and Polyplacidae (outgroup species *Fahrenholzia pinnata* and *F. reducta*; Table 1). Multiple samples of *Pediculus humanus* were included in this study to represent both head and body lice as well as lice belonging the mitochondrial Clades A and B identified in Reed et al. (2004). Lice were preserved in 95% ethanol and stored at −80 °C. DNA was isolated from louse specimens using the DNAeasy Tissue Kit (QIAGEN Inc., Valencia, California) using louse specific protocols (Cruickshank et al., 2001; Johnson and

Table 1
Louse specimens included in phylogenetic and dating analyses.

Louse family and species	Louse voucher ID	Collection locality	Host identification
Pediculidae			
Pediculus humanus capitis 3[a]	Pdcap3.27.06.3	USA: Utah: Salt Lake City	*Homo sapiens* (SLC 2,7,7)
Pediculus humanus capitis 20[a]	Pdcap9.20.05.20	USA: Florida: West Palm Beach	*Homo sapiens* (WP016)
Pediculus humanus capitis 23[b]	Pdcap9.20.05.23	USA: Florida: West Palm Beach	*Homo sapiens* (WP012)
Pediculus humanus capitis 25[b]	Pdcap9.20.05.25	USA: Florida: West Palm Beach	*Homo sapiens* (WP007)
Pediculus humanus humanus 1[a]	Pdhum11.19.02.1	Canada: Lethbridge	*Homo sapiens*
Pediculus humanus humanus 3[a]	Pdhum9.6.06.3	Burundi	*Homo sapiens*
Pediculus schaeffi	Pdsch5.23.05	Uganda	*Pan troglodytes*
Pthiridae			
Pthirus gorillae	Ptgor8.1.06.6	Uganda	*Gorilla gorilla* (MGVP 051122CAWBB001)
Pthirus pubis	Ptpub1.19.06.3	UK: Scotland: Glascow	*Homo sapiens* (GLA 140)
Pedicinidae			
Pedicinus badii	Qnbad7.24.06.9	Uganda	*Procolobus rufomitratus* (999)
Polyplacidae			
Fahrenholzia pinnata	Fzpin163.1	USA: Nevada: Tonopah	*Perognathus longimembris* (MLZ 2039)
Fahrenholzia reducta	Fzred7.24.06.10	USA: California: Red Mountain	*Chaetodipus formusus* (MLZ 1863)

Abbreviations used: MLZ, Moore Laboratory of Zoology; Page Lab; GLA, University of Glasgow; Lice Solutions; WP, West Palm; MGVP, Maryland Gorilla Veterinary Project.
[a] These lice belong to mitochondrial Clade A, consisting of both head and body lice (Reed et al., 2004).
[b] These lice belong to mitochondrial Clade B, consisting only of head lice (Reed et al., 2004).

Clayton, 2003). After DNA extraction, lice were mounted on slides and retained as vouchers. Voucher specimens will be deposited in the Price Institute for Phthirapteran Research collection (University of Utah).

Portions of the mitochondrial genes COI, cytochrome *b* (Cytb), and cytochrome *c* oxidase subunit 3 (CO3), and a portion of the nuclear genes 18S rRNA, EF-1α, rudimentary (CAD), RNA polymerase II (Pol II), wingless (Wg), and histone 3 (H3) were amplified and sequenced with primers listed in Table 2. With the exception of 18S rRNA, all genes are protein-coding. Double-stranded PCR amplifications for all molecular markers were performed in 25 μl reaction volumes using 10 μl of Eppendorf HotMaster PCR Mix (Fisher Scientific), 1 μl of each primer (at 10 mM; Table 2), and 2 μl of DNA template. The amplification protocol for COI, Cytb, CO3, and EF-1α required an initial denaturation step of 94 °C for 10 min, followed by 5 cycles of 94 °C (1 min), 48 °C (1 min), and 65 °C (2 min), then 30 cycles of 94 °C (1 min), 52 °C (1 min), and 65 °C (2 min) and a final extension of 65 °C for 10 min. Amplification of a portion of the nuclear 18S rRNA gene was performed using the primers 18Sai and 18Sbi (Whiting et al., 1997), NS1 and NS2a (Barker et al., 2003), NS5a (Barker et al., 2003) and NS8 (Black et al., 1997), and 18S680f and 18S2875r according to the cycling protocol listed above. The other 18S rRNA primers listed in Table 2 were used as internal sequencing primers. The amplification protocol for the other nuclear markers required an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 94 °C (1 min), annealing temperature (55 s), and 65 °C (1 min) and a final extension of 65 °C for 5 min where the annealing temperatures were 52, 49, 46, and 54 °C for CAD, Pol II, Wg and H3, respectively. In the event of PCR failure, lower annealing temperatures were employed. Amplified products were purified using ExoSAP-IT (USB Corporation) or the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California; this method was used primarily for Wg) and sequenced in both directions. All sequencing reactions were performed at the University of Florida DNA Sequencing Core Laboratory using ABI Prism BigDye Terminator cycle sequencing protocols (Applied Biosystems; Perkin-Elmer Corp., Foster City, CA). Excess dye-labeled terminators were removed by ethanol precipitation and purified products were dried using SpeedVac® (ThermoSavant, Holbrook, NY, USA) and suspended in Hi-di formamide. Sequencing reactions were performed using POP-7 sieving matrix on 50-cm capillaries in an ABI Prism® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by ABI Sequencing Analysis software v.5.2 and KB Basecaller.

Sequences were edited using Sequencher v.4.2.2 (Gene Codes Corporation, Ann Arbor, Michigan) and all markers (except 18S rRNA, see below) were aligned by eye using Se-Al v2.0a11 (Rambaut, 1996). Primer sequences were removed and sequences were trimmed in reference to the translated protein sequence using Se-AL v2.01a11 and MacClade 4.0 (Maddison and Maddison, 2000). Louse 18S rRNA sequences were aligned manually in reference to secondary structure (Gillespie, 2004; Gillespie et al., 2005; alignment available at the jRNA web site http://hymenoptera.tamu.edu/rna/models/arth/data/alignment/18S_arthropods.00.04.Nex) and all ambiguously aligned sites were removed from the alignment before analysis. All sequences are available on GenBank (EF152552–

Table 2
Names, sequences, and references of genes and primers.

Gene	Primer	Sequence (5′→3′)	Source
COI	LCO1718	GGRGGRTTYGTAAATTGRYTWRTTCC	Reed et al. (2004)
	H7005	CCGGATCCACNACRTARTANGTRTCRTG	Hafner et al. (1994)
Cytb[a]	L11122	GAAATTTTGGGTCWTTRCTNGG	Reed et al. (2004)
	H11823	GGCATATGCGAATARGAARTATCA	Reed et al. (2004)
CO3[b]	HCOT51	CAYCCATTTCATNTNGTNGAT	This study[c]
	HCOT162	TGGTGACGAGAYGTKAYTCGAGA	This study[c]
	C3-N-5460	TCAACAAAGTGTCAGTATCA	Simon et al. (1994)
18S rRNA	18Sai	CCTGAGAAACGGCTACCACATC	Whiting et al. (1997)
	18Sbi	GAGTCTCGTTCGTTATCGGA	Whiting et al. (1997)
	NS1	GTAGTCATATGCTTGTCTC	Barker et al. (2003)
	NS2a	CGCGGCTGCTGGCACCAGACTTGC	Barker et al. (2003)
	NS5a	TGAAACTTAAAGGAATTGACGGAAG	Barker et al. (2003)
	NS8	TCCGCAGGTTCACCTACGGA	Black et al. (1997)
	18S680f	ATTGGAGGGCAAGTCTGG	This study
	18S1852f	CATTAGTTATTGCGGCGTTAG	This study
	18S2490r	CTAAGGGCATCACAGACCTGTTA	This study
	18S2875r	CCTACGGAAACCTTGTTACGAC	This study
	18S3095r	CAGGCTAGAGTCTCCATCG	This study
EF-1α[d]	EF1For3	AGCCTCTTCGACTGCCATTA	Danforth and Ji (1998)
	Cho10	ACRGCVACKGTYTGHCKCATGTC	Danforth and Ji (1998)
CAD[e]	ApCADfor1	GGWTATCCCGTDATGGCBMGWGC	Danforth et al. (2006)
	Ap835rev1	GCATHACYTCHCCCACRCTYTTC	Danforth et al. (2006)
Pol II	polfor2	TGGGAYGSYAAAATGCCKCAACC	Danforth et al. (2006)
	polrev2	TTYACAGCAGTATCRATRAGACCTTC	Danforth et al. (2006)
	RPII735f	CAGGTTATTGCYTGYGTNGGTCA	This study
	RPII1152r	AARTAYCTTTCRTTYGTNGSATCAAA	This study
Wg[f]	LepWg1	GARTGYAARTGYCAYGGYATGTCTGG	Brower and DeSalle (1998)
	ModLepWg2	ACTICGCARCACCARTGGAATGTRCA	Brower and DeSalle (1998)
H3[f]	H3AF	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. (1998), Terry and Whiting (2005)
	H3AR	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (1998), Terry and Whiting (2005)

See text for definition of gene abbreviations.
[a] PCR amplification of Cytb was unsuccessful for *Fahrenholzia pinnata*, *F. reducta*, and *Pthirus gorillae*.
[b] PCR amplification of CO3 was unsuccessful for *Fahrenholzia pinnata*, *F. reducta*, and *Pedicinus badii*.
[c] CO3 sequences designed using GenBank Accession No. DQ054849 (Covacin et al., 2006).
[d] PCR amplification of EF-1α was unsuccessful for *Pediculus humanus humanus 1* (Table 1).
[e] PCR amplification of CAD was unsuccessful for *Fahrenholzia reducta*.
[f] PCR amplification of Wg and H3 was unsuccessful for *Fahrenholzia pinnata*.

EF152561, EF152563–EF152564, EU493445–EU493447, FJ267392–FJ267475).

2.2. Phylogenetic analysis

Prior to phylogenetic analyses, base composition bias was evaluated for each marker across all taxa using Chi-square (χ^2) goodness-of-fit tests in PAUP*b4.10 (Swofford, 2003). Phylogenetic signal of each data set also was assessed in PAUP*b4.10 (Swofford, 2003) using the *g*-statistic following the procedures of Hillis and Huelsenbeck (1992) as well as the permutation tail probability test (PTP-test; Faith, 1991; Faith and Cranston, 1991). Lastly, sequence divergence among taxa was determined using uncorrected *p*-distances in PAUP*b4.10 (Swofford, 2003).

Phylogenetic congruence of the nine molecular markers was evaluated using the partition homogeneity test (PHT- or ILD-test; Farris et al., 1994) in PAUP*4.0b10 (Swofford, 2003). One thousand partition replicates were analyzed by maximum parsimony (heuristic search option and random addition replicates of tree bisection–reconnection branch swapping). The ILD-test detected significant heterogeneity among the nine molecular markers ($P \leqslant 0.008$), and it was only when H3 was removed from the data set that the ILD-test was not significant ($P > 0.2$). Therefore, along with analyzing each gene individually, we also combined all genes into a single matrix and examined this matrix both including (9-gene data set) and excluding (8-gene data set) H3.

Phylogenetic analyses of individual and combined genes were performed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian approaches. Equally weighted MP searches were performed with 10 random addition replicates and tree bisection–reconnection branch swapping using PAUP*4.0b10 (Swofford, 2003). To assess nodal support, nonparametric bootstrap analyses were performed (500 pseudoreplicates and 10 random sequence additions; Felsenstein, 1985). To generate the best ML and Bayesian trees, Modeltest (Version 3.7; Posada and Crandall, 1998) and MrModelTest (Nylander, 2004) were used to examine models of nucleotide substitution (56 and 24, respectively) and choose a best-fit model of sequence evolution. Models of evolution providing the best approximation of the data using the fewest parameters were chosen for subsequent analyses according to the Akaike Information Criterion (Huelsenbeck and Rannala, 1997; Posada and Buckley, 2004). Nucleotide substitution models for each data set are given in Table 3. Full heuristic ML and bootstrap (200 pseudoreplicates) searches were conducted using the best-fit model in PAUP*4.0b10 (Swofford, 2003).

Bayesian phylogenetic analyses were performed in MrBayes 3.12 (Huelsenbeck and Ronquist, 2001) on the combined 9- and 8-gene data sets only using the best-fit substitution described above (Table 3). Model parameters were treated as unknown variables with uniform priors and were estimated as part of the analysis. Bayesian analyses were initiated from random starting trees, run for 10 million generations with 4 incrementally heated chains (Metropolis-coupled Markov chain Monte Carlo; Huelsenbeck and Ronquist, 2001), and sampled at intervals of 1000 generations. Two independent Bayesian analyses were run to avoid entrapment on local optima, and log-likelihood scores were compared for convergence so that burn-in generations (the first 3000 trees) could be discarded. Tracer v1.4 (Rambaut and Drummond, 2004) was used to evaluate stability of all parameter estimates following removal of burn-in generations. The 9- and 8-gene data sets also were examined with partitioned Bayesian phylogenetic analyses. Individual genes were defined as partitions *a priori* and each partition was assigned its own substitution model according to MrModelTest (Table 3). Partitioned Bayesian analyses were performed as described above.

Bayesian partitioning schemes (i.e., partitioned versus non-partitioned) were assessed using Bayes factors (Nylander et al., 2004). Bayes factors were computed using the harmonic means of the likelihoods calculated from the *sump* command within MrBayes. A difference of 2ln Bayes factor >10 was used as the minimum value to discriminate between analysis schemes (Brandley et al., 2005; Brown and Lemmon, 2007). Alternative phylogenetic hypotheses were compared statistically using the Kishino–Hasegawa (KH) and the Shimodaira–Hasegawa tests (SH) as implemented in PAUP*4.0b10 (MP and ML analyses using RELL optimization and 1000 bootstrap replicates; Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999; Goldman et al., 2000). All executable files are available on TreeBase (http://www.treebase.org; Submission ID SN4122; Study Accession No. S2207).

2.3. Estimates of divergence times

Divergence times were estimated using Bayesian approaches implemented in *multidivtime* (Kishino et al., 2001; Thorne and Kishino, 2002) and BEAST v1.46 (Drummond and Rambaut, 2007) by analyzing genes individually as well as in a combined framework. Before estimating divergence times, likelihood ratio

Table 3
Data set properties and substitution models used in phylogenetic and divergence time analyses.

Data set	Substitution model	Fragment length	PI[a] Sites	Number of tree[b]	Tree length	CI[c]	RI[d]	Clock?[e]
COI	GTR + I + G	858	333	4	959	0.717	0.643	Yes
Cytb	HKY + I + G	699	166	4	590	0.863	0.701	No
CO3	GTR + I	525	180	1	387	0.935	0.904	Yes
18S rRNA	GTR + I + G	1804	45	18	173	0.936	0.879	No
EF-1α	GTR + I	345	75	2	149	0.831	0.838	Yes
CAD	GTR + I + G	612	132	2	310	0.855	0.796	Yes
Pol II	GTR + I	393	96	2	203	0.818	0.794	Yes
Wg	GTR + I + G	384	69	1	172	0.855	0.811	No
H3	GTR + I	327	70	1	186	0.828	0.740	No
9-gene	GTR + I + G	5947	1166	1	3142	0.820	0.741	No
8-gene[f]	GTR + I + G	5620	1096	1	2947	0.822	0.746	No

[a] Number of potentially parsimonious informative sites.
[b] Number of equally parsimonious trees.
[c] Consistency index.
[d] Retention index.
[e] Do sequence data depart significantly from clock-like behavior?.
[f] The 8-gene data set does not include H3 (see text).

tests were performed using PAUP*4.0b10s (Swofford, 2003) to determine if the sequence data departed significantly from clock-like behavior. Because primates and their ectoparasitic sucking lice are known to cospeciate (Reed et al., 2004, 2007), speciation events in these taxa are assumed to be roughly contemporaneous. The louse tree can therefore be calibrated by placing primate fossil dates on the corresponding node of the louse tree. For all data sets, a basal calibration of 20–25 Ma (corresponding to the split between Old World monkeys [OWM] and apes; Maclatchy, 2004; Steiper et al., 2004; Young and MacLatchy, 2004; Kumar et al., 2005) was used to represent the split between Old World monkey lice (*Pedicinus* spp.) and hominoid lice (*Pthirus* spp. and *Pediculus* spp.). Additionally, a terminal calibration of 5–7 Ma (corresponding to the split between chimpanzees and humans; Stauffer et al., 2001) was used to represent the split between human *Pediculus* lice (*Pediculus humanus*) and the chimp louse (*Pediculus schaeffi*). These two calibration points were used in combination as well as individually to cross-check the other calibration point, and calibrations were treated as fixed minimuma and maximuma in *multidivtime* or assigned a normal prior distribution in BEAST (see below). Divergence times were estimated for the following nodes in Fig. 1: (1) the split between Old World monkey lice (*Pedicinus*) and hominoid lice (*Pthirus* + *Pediculus*), (2) the split between *Pthirus* and *Pediculus*, (3) the split between *Pthirus gorillae* and *Pthirus pubis*, (4) the split between *Pediculus humanus* and *Pediculus schaeffi*, and (5) the age of *Pediculus humanus*. Timing of divergences within *Pediculus humanus* also were estimated, specifically the age of lice belonging the mitochondrial Clade A (Node 6, head and body lice) and Clade B (Node 7, head lice only; Fig. 1) defined by Reed et al. (2004). To properly estimate divergence times at these nodes, the topology resulting from phylogenetic analysis of the combined 9- and 8-gene data sets was defined *a priori* in all dating analyses. This tree topology supports monophyly of each of the 7 nodes defined above (see below) and is in agreement with previous primate louse studies (Fig. 1; Reed et al., 2004, 2007).

The parametric Bayesian (PB) approach of Thorne and Kishino (2002) is able to accommodate multiple loci with variable evolutionary characteristics and the molecular data from each gene were treated as separate data partitions. For PB analyses, model parameters for the F84 + Γ model were estimated for each gene partition using the *baseml* program in PAML v3.14 (Yang, 1997). These parameters were used in the program *estbranches* (Kishino et al., 2001; Thorne and Kishino, 2002) to estimate the ML and variance–covariance matrix (also using the F84 + Γ model) of the branch length estimates. Lastly, the program *multidivtime* (Kishino et al., 2001; Thorne and Kishino, 2002), utilizing the output files from *estbranches* and implementing Markov chain Monte Carlo sampling, was used to estimate prior and posterior distribution of both the substitution rates and the estimated divergence time for each node. The prior assumption for the mean and standard deviation of the time of the ingroup root node (rttm) was set to 3.0 time units, where 1 time unit represents 10 million years for the maximum age for ingroup root node. The mean and standard

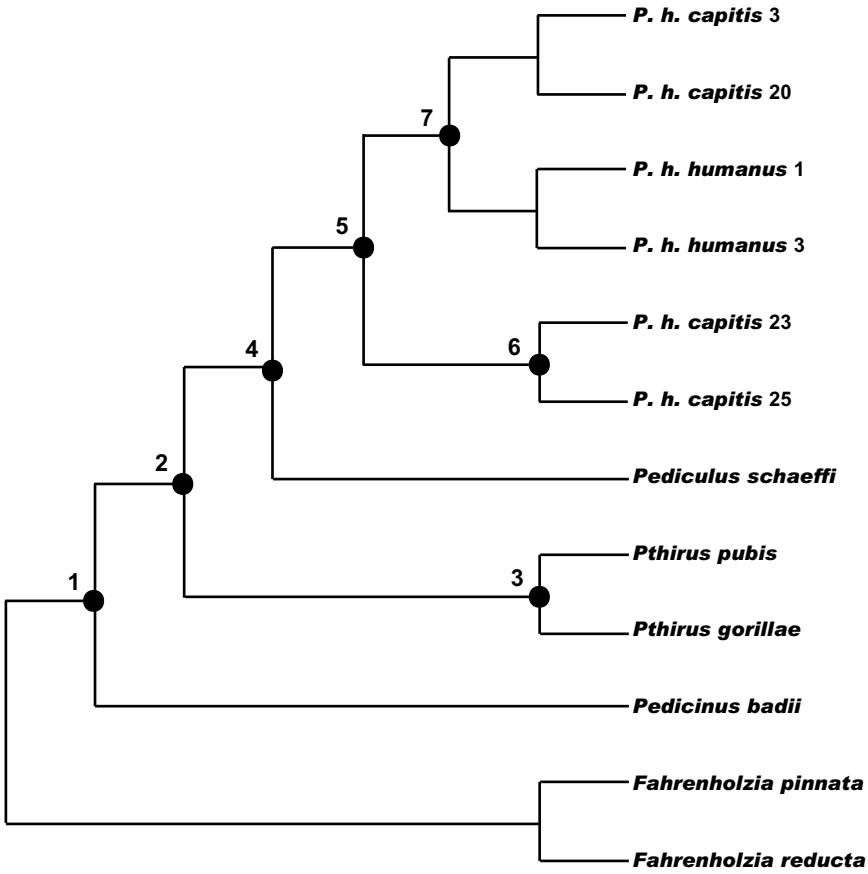

Fig. 1. Primate louse topology determined in Reed et al. (2007). Node numbers are as follows: (1) the split between Old World monkey lice (*Pedicinus*) and hominoid lice (*Pthirus* + *Pediculus*), (2) the split between *Pthirus* and *Pediculus*, (3) the split between *Pthirus gorillae* and *Pthirus pubis*, (4) the split between *Pediculus humanus* and *Pediculus schaeffi*, (5) the age of *Pediculus humanus*, and *Pediculus humanus* mitochondrial Clades A (Node 6) and B (Node 7). All nodes were constrained to be monophyletic in estimates of divergence times. Abbreviations are as follows: *Pediculus humanus* (*P. h.*).

deviation for the prior distribution of the rate of evolution at the ingroup node (rtrate and rtratesd) was determined following the procedure of Jansa et al. (2006). The basal and terminal nodal calibrations described above were applied on the appropriate tree node as minimum and maximum bounds. The Markov chain was initialized by randomly selecting the initial parameter value and each chain was sampled every 100 cycles for 10^6 generations with a burn-in of 10^5 cycles. Analyses were performed twice to ensure stationarity.

BEAST v1.46 (Drummond and Rambaut, 2007) also was used to estimate divergence times using the relaxed phylogenetics method of Drummond et al. (2006). BEAST uses a Bayesian relaxed molecular clock while incorporating tree uncertainty in the MCMC process to infer divergence times. A Yule process speciation prior and an uncorrelated log-normal model of rate variation were implemented in each analysis (Drummond et al., 2006). Node constraints were assigned a normal prior distribution with means equal to 22.5 and 6 for the basal OWM-ape and terminal chimp-human splits, respectively, with the standard deviations encompassing the minimum and maximum age of each calibration. A normal distribution was chosen because it allows uncertainty in the calibration estimates (Ho, 2008), which is important for our data because calibrations were taken from the primate fossil record rather than from louse fossils (a sufficient louse fossil record is lacking; Light and Hafner, 2008, and references therein). Posterior probability distributions of node ages were obtained for each gene separately and the combined 9- and 8-gene alignments using a user-supplied tree. In the 9- and 8-gene alignments, the genes were analyzed in a concatenated and partitioned framework (model parameters were unlinked across partitions). Best-fit models of nucleotide substitution for each data set were the same as those identified above as part of the phylogenetic analyses using Modeltest (Posada and Crandall, 1998) and MrModelTest (Nylander, 2004; Table 3). After an initial period of fine-tuning the operators, two separate MCMC analyses were run for 30,000,000 generations (burn-in 10%) with parameters sampled every 1000 steps. Independent runs were combined using LogCombiner v.1.4.6 (Drummond and Rambaut, 2007). Tracer v1.4 was then used to measure the effective sample size of each parameter (all resulting effective sample sizes exceeded 100) and calculate the mean and upper and lower bounds of the 95% highest posterior density interval (95% HPD) for divergence times (Rambaut and Drummond, 2004). Tree topologies were assessed using TreeAnnotator v.1.4.6 (Drummond and Rambaut, 2007) and FigTree v.1.1.2 (Rambaut, 2008). Bayes factors of non-partitioned and partitioned 9- and 8-gene data sets were assessed using Tracer v1.4 (Suchard et al., 2001).

Table 4
Mean pairwise uncorrected *p*-distances (in percentages) for each gene among all Anoplura taxa, representing the families Pediculidae, Pthiridae, Pedicinidae, and Polyplacidae, as well as only among ingroup (primate lice) taxa.

	All Anoplura taxa	Primate louse taxa
COI	21.87	17.67
Cytb	22.36	18.94
CO3	21.70	21.70
18S rRNA	3.74	3.00
EF-1α	11.60	8.08
CAD	11.93	9.97
Pol II	11.80	7.83
Wg	10.80	7.99
H3	12.82	10.50

3. Results

3.1. Phylogenetic analyses

None of the louse taxa examined showed significant departures from expected base composition ($\chi^2 < 17.18$, df = 3) and each data set exhibited high levels of phylogenetic signal (i.e., nonrandom structure) according to the *g*-statistic of Hillis and Huelsenbeck (1992; $P < 0.05$; data not shown) and the PTP-test (1000 replicates, $P < 0.001$). Mean pairwise sequence divergences (uncorrected *p*-distances) were variable among genes, ranging from 3.74% (3.00% ingroup taxa only) to 22.36% (18.94% ingroup taxa only) for 18S rRNA and Cytb, respectively (Table 4). Sequence divergence for the other nuclear markers (EF-1α, CAD, Pol II, Wg, and H3) were in between divergences for the slow-evolving 18S rRNA and fast-evolving mitochondrial markers (Table 4).

The nine genes examined in this study provided different levels of phylogenetic resolution among primate lice (Table 3 and Fig. 2). For each gene, MP and ML phylogenetic analyses yielded identical trees with similar support values, although nodal support varied depending on the gene examined (Fig. 2). Most topologies of individual genes were in agreement with previous studies of primate lice (Reed et al., 2007; Fig. 1), supporting monophyly of both *Pediculus* and *Pthirus* as well as a sister relationship between these two genera. Phylogenetic analysis of the nuclear coding genes Pol II (Fig. 2g) and H3 (Fig. 2i), however, resulted in tree topologies that were in conflict with the other gene trees. Analyses constraining a sister relationship between *Pediculus* and *Pthirus* in Pol II resulted in a tree that was not significantly different from the best tree based on MP and ML Kishino–Hasegawa tests (KH tests; $P > 0.6$) or the Shimodaira–Hasegawa test (SH tests; $P > 0.3$). For H3, however, analyses constraining a monophyletic *Pediculus* and a sister relationship between *Pediculus* and *Pthirus* yielded a tree that was significantly worse than the best tree (KH and SH tests $P < 0.03$).

Analysis of the concatenated 9- and 8-gene data sets using MP, ML, or Bayesian approaches resulted in identical topologies with similar nodal support (Fig. 3). This topology is consistent with the gene trees presented in Fig. 2 (except for Pol II and H3; see above), as well as previous studies of primate lice (Reed et al., 2007; Fig. 1). Although Bayes factors indicated that a partitioned scheme is preferred over a non-partitioned scheme, partitioned analyses of both the 9- and 8-gene data set yielded the same topology and support values as in the non-partitioned Bayesian analyses. Support values at one node (*Pediculus humanus capitis* 3 and *Pediculus humanus capitis* 20), however, were much higher in the partitioned analyses (0.99; data available upon request).

3.2. Estimates of divergence times

The molecular clock was rejected in six of the 11 data sets (Table 3; also verified in BEAST analyses, data available upon request), thus the most appropriate divergence dating techniques are those that relax a molecular clock (i.e., *multidivtime* and BEAST; Thorne and Kishino, 2002; Drummond et al., 2006). Because Cytb and CO3 could only be collected for 9 taxa (Table 2) and because a constrained topology for H3 was significantly worse than the best tree, these genes were not examined individually in estimates of divergence times (they were, however, included in the combined analyses). Mean divergence time estimates did not differ significantly between the two dating techniques, although the credibility intervals in *multidivtime* were generally larger than the BEAST 95% HPD (Appendices A and B). Notably, there were large differences in divergence estimates

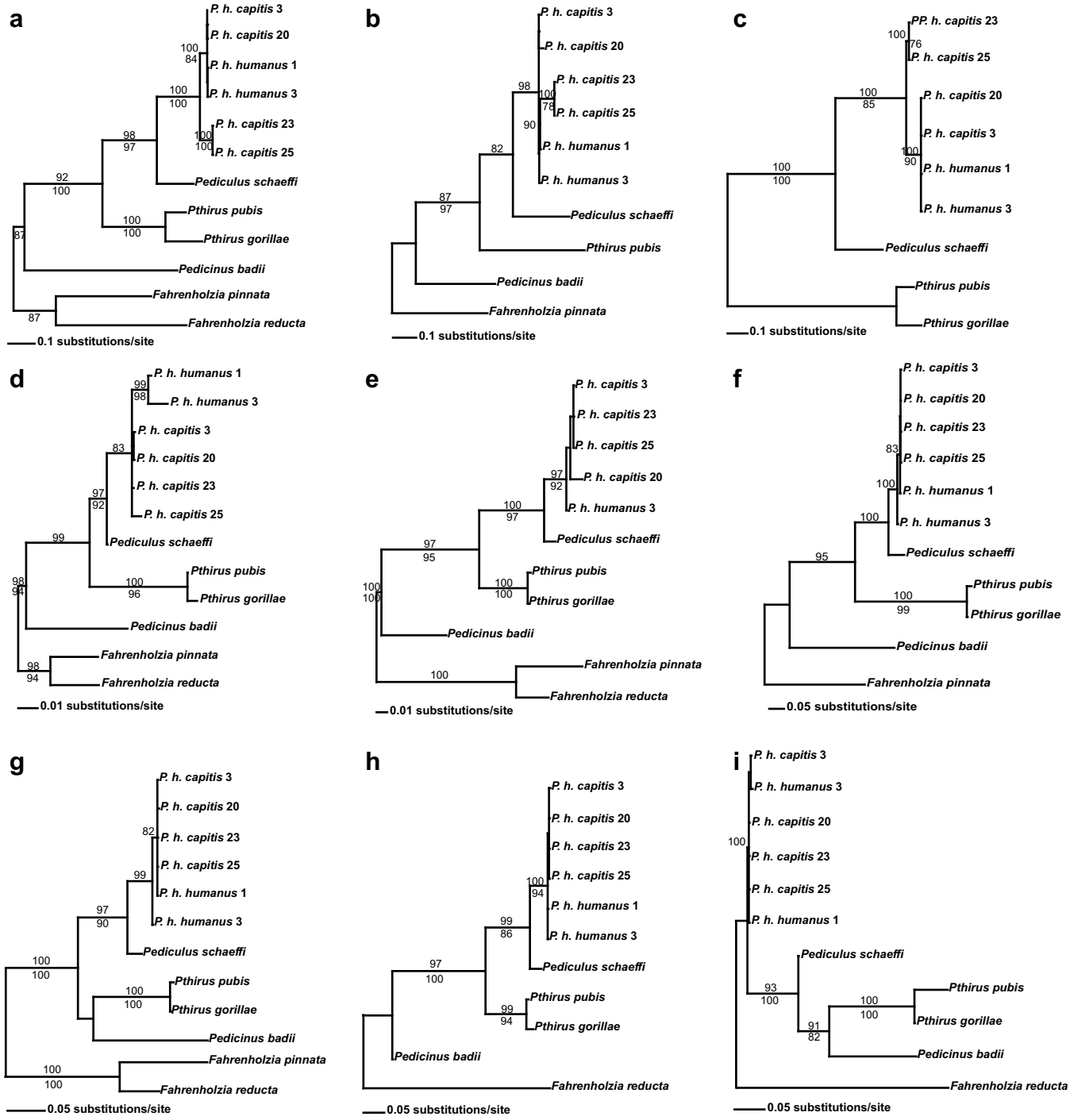

Fig. 2. Maximum-likelihood (ML) phylograms resulting from analysis of each gene: (a) COI, (b) Cytb, (c) CO3, (d) 18S rRNA, (e) EF-1α, (f) CAD, (g) Pol II, (h) Wg, and (i) H3 (see text for full gene names). Parsimony and ML bootstrap support values greater than 75 are located above and below the nodes, respectively. Abbreviations of scientific names are as in Fig. 1.

among data sets and calibrations. Estimates tended to be more variable for the nuclear loci and when estimated nodes were farther from calibration points (Appendices A and B). Additionally, analyses using only the basal calibration (OWM-ape divergence of 20–25 Ma; Node 1) tended to overestimate the age of the terminal chimp-human split (Node 4) whereas analyses only using the terminal calibration tended to underestimate the age of the OWM-ape split. This was especially obvious when genes were analyzed individually. Genes analyzed in combination (9- and 8-gene data sets, partitioned and non-partitioned) produced less variable estimates than those produced by individual markers. According to Bayes factors, partitioned data sets are the preferred partitioning scheme, although analyses of partitioned and non-partitioned data sets produced similar results (Table 5). Estimates from 9- and 8-gene partitioned data sets, regardless of methodology or calibration, are in agreement with the results of Reed et al. (2007; Fig. 4). We find that *Pthirus gorillae* and *Pthirus pubis* diverged approximately 3 Ma (Fig. 4, Node 3; Table 5), supporting a recent host switch of *Pthirus* from gorillas to humans. We also find that *Pediculus* and *Pthirus* diverged

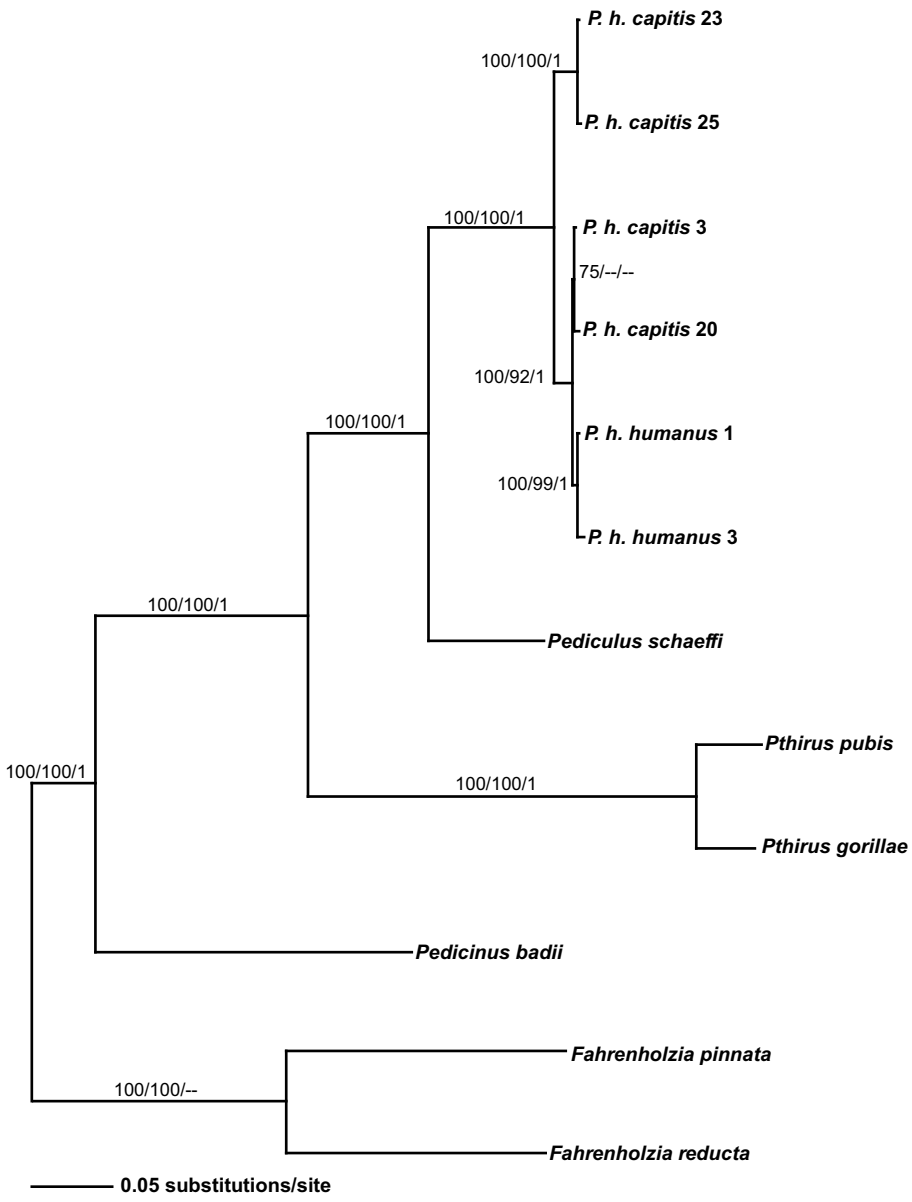

Fig. 3. Maximum-likelihood (ML) phylogram resulting from the analysis of the combined 9-gene data set. Parsimony and ML bootstrap support values greater than 75 and Bayesian posterior probabilities resulting from the non-partitioned analysis greater than 0.95 are located above the nodes. Bayesian posterior probabilities were identical in the 9- and 8-gene data sets. Abbreviations of scientific names are as in Fig. 1.

13 Ma (Fig. 4, Node 2; Table 5) supporting an ancient louse duplication event on the common ancestor of humans, chimps, and gorillas (Reed et al., 2007).

4. Discussion

4.1. Phylogenetic analysis of molecular markers

Although lice are model organisms in cospeciation studies and may thus facilitate inferences of host evolutionary history, few molecular markers are available to construct louse phylogenies. Currently published tree topologies for lice may therefore represent unique evolutionary events for a particular gene (i.e., incomplete lineage sorting, gene duplication and extinction, hybridization, or lateral gene transfer) rather than species relationships (Maddison, 1997). Analysis of multiple genes in a concatenated framework can increase confidence that the resulting phylogeny represents the species tree (Chen and Li, 2001; Rokas et al., 2003; Driskell et al., 2004; Gadagkar et al., 2005) as well as identify markers that can be phylogenetically informative in other taxonomic groups. One of the goals of this study was to build a more robust phylogeny of primate louse relationships and, in doing so, identify molecular markers that could be useful for parasite systematics. Before performing a combined analysis the nine genes amplified in this study, each marker was first assessed individually to identify possible sources of topological incongruence among loci as well as characterize the potential utility of these markers for future studies.

Of the mitochondrial genes examined in this study, COI is one of the most commonly used in louse, as well as insect, phylogenetic studies. It is a fast-evolving marker (Table 4) and has been especially useful for species-level phylogenies (see references above). We find that COI is phylogenetically informative not only among species, but also among louse genera and families (Fig. 2a). How-

Table 5
Divergence times for primate louse clades based on *multidivtime* and BEAST analyses of combined data sets. Values shown are the average and 95% credibility intervals (in parentheses) and are the posterior mean ages and 95% HPD (in parentheses) for *multidivtime* and BEAST, respectively. See text and Fig. 1 for data set and Node definitions.

Data set	Node 1	Node 2	Node 3	Node 4	Node 5	Node 6	Node 7
Basal calibration (Node 1)[a]							
Multidivtime							
9-Gene	22.80 (20,18, 24.91)	15.13 (11.76, 18.69)	2.77 (1.61, 4.29)	7.36 (4.99, 10.30)	1.23 (0.71, 2.00)	0.12 (0.03, 0.27)	0.07 (0.00, 0.17)
8-Gene	22.79 (20.20, 24.91)	15.04 (11.65, 18.64)	2.90 (1.66, 4.51)	7.23 (4.88, 10.07)	1.28 (0.72, 2.03)	0.12 (0.03, 0.27)	0.07 (0.00, 0.18)
BEAST							
9-Gene	23.30 (19.33, 25.23)	14.40 (9.61, 18.92)	3.26 (1.07, 6.24)	7.48 (3.68, 11.98)	1.74 (0.57, 3.43)	0.43 (0.18, 0.79)	0.13 (0.02, 0.29)
9-Gene part[b]	22.30 (19.30, 25.23)	13.49 (8.94, 18.04)	3.00 (0.97, 6.13)	6.39 (3.03, 10.74)	1.38 (0.45, 2.81)	0.34 (0.13, 0.65)	0.11 (0.02, 0.24)
8-Gene	22.28 (19.35, 25.24)	14.27 (9.39, 18.90)	3.38 (0.95, 6.77)	7.24 (3.26, 12.00)	1.91 (0.59, 4.08)	0.46 (0.16, 0.97)	0.15 (0.02, 0.36)
8-Gene part[b]	22.30 (19.40, 25.30)	13.46 (8.06, 18.77)	3.04 (0.80, 6.58)	6.39 (2.65, 11.52)	1.66 (0.36, 3.66)	0.50 (0.09, 0.98)	0.19 (0.02, 0.32)
Terminal calibration (Node 4)[c]							
Multidivtime							
9-Gene	22.41 (16.40, 29.93)	13.80 (10.46, 17.75)	2.49 (1.41, 3.98)	6.13 (5.07, 6.96)	1.02 (0.66, 1.47)	0.10 (0.03, 0.21)	0.05 (0.00, 0.14)
8-Gene	22.89 (16.63, 31.07)	14.04 (10.59, 18.15)	2.68 (1.51, 4.25)	6.14 (5.08, 6.96)	1.07 (0.69, 1.56)	0.10 (0.03, 0.22)	0.05 (0.00, 0.13)
BEAST							
9-Gene	17.69 (8.41, 29.19)	11.48 (6.17, 18.47)	2.63 (0.66, 5.21)	5.91 (4.92, 6.91)	1.42 (0.43, 2.85)	0.37 (0.11, 0.75)	0.11 (0.02, 0.27)
9-Gene part[b]	20.67 (9.50, 32.94)	12.50 (6.75, 19.50)	2.71 (0.69, 5.30)	5.92 (4.91, 6.89)	1.25 (0.35, 2.45)	0.31 (0.10, 0.60)	0.09 (0.02, 0.21)
8-Gene	18.48 (8.89, 30.02)	11.76 (6.54, 18.50)	2.87 (0.63, 5.86)	5.91 (4.96, 6.93)	1.55 (0.46, 3.24)	0.39 (0.11, 0.84)	0.13 (0.01, 0.31)
8-Gene part[b]	21.73 (9.66, 35.13)	12.90 (6.88, 20.43)	2.70 (0.70, 5.50)	5.91 (4.90, 6.88)	1.23 (0.42, 2.38)	0.30 (0.09, 0.58)	0.10 (0.01, 0.21)
Basal and terminal calibrations							
Multidivtime							
9-Gene	22.29 (20.11, 24.76)	13.93 (11.52, 16.51)	2.54 (1.52, 3.85)	6.23 (5.15, 6.97)	1.04 (0.67, 1.49)	0.10 (0.03, 0.21)	0.05 (0.00, 0.14)
8-Gene	22.36 (20.13, 24.82)	14.00 (11.50, 16.55)	2.71 (1.61, 4.11)	6.20 (5.15, 6.96)	1.09 (0.70, 1.55)	0.10 (0.03, 0.22)	0.06 (0.00, 0.14)
BEAST							
9-Gene	22.10 (19.21, 25.00)	13.84 (9.36, 18.27)	3.07 (0.90, 5.66)	6.04 (5.11, 6.97)	1.57 (0.66, 2.90)	0.41 (0.17, 0.75)	0.13 (0.03, 0.28)
9-Gene part[b]	22.31 (19.38, 25.14)	13.37 (9.51, 17.43)	2.80 (1.03, 5.21)	5.96 (5.03, 6.91)	1.31 (0.46, 2.47)	0.33 (0.13, 0.55)	0.10 (0.02, 0.21)
8-Gene	22.13 (19.22, 25.00)	13.76 (9.33, 18.13)	3.17 (0.99, 6.03)	6.03 (5.12, 7.01)	1.69 (0.61, 3.29)	0.43 (0.17, 0.83)	0.13 (0.02, 0.31)
8-Gene part[b]	22.38 (19.51, 25.25)	13.18 (9.43, 16.93)	2.77 (1.06, 5.13)	5.94 (4.97, 6.86)	1.23 (0.52, 2.28)	0.28 (0.13, 0.51)	0.09 (0.02, 0.19)

[a] Basal calibration of 20–25 Ma at Node 1 corresponding to the split between old world monkeys and apes.
[b] 9-gene and 8-gene data sets were partitioned in BEAST.
[c] Terminal calibration of 5–7 Ma at Node 4 corresponding to the split between chimpanzees and humans.

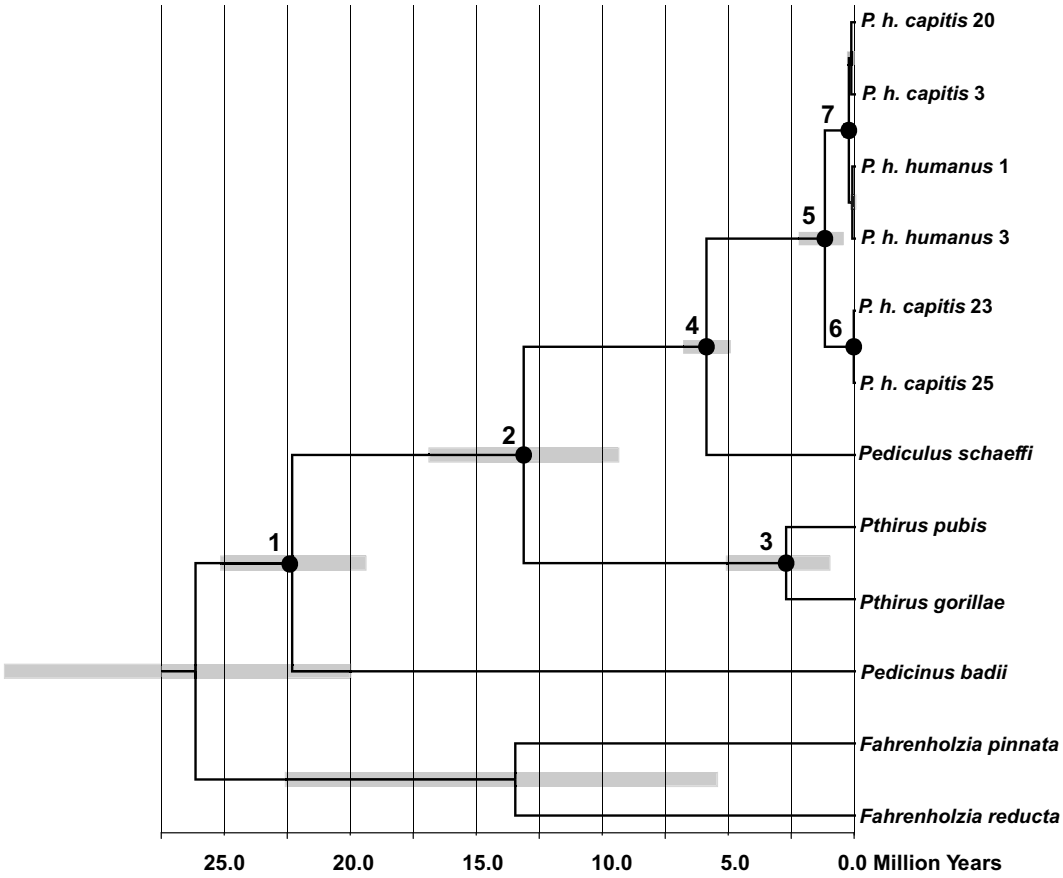

Fig. 4. Divergence time estimation (in millions of years) for primate lice. Shown is the Bayesian topology resulting from analysis of the 8-gene partitioned data set in BEAST (Drummond and Rambaut, 2007). Nodes are numbered as in Fig. 1 and circles at nodes indicate mean ages whereas gray bars indicate the upper and lower bounds of the 95% highest posterior density interval (95% HPD). Abbreviations of scientific names are as in Fig. 1.

ever, it should be noted that the outgroup taxa in our study (Polyplacidae: *Fahrenholzia*) are closely related to primate lice (J.E. Light, unpublished data) and phylogenetic resolution in COI may deteriorate as more distantly related Anoplura taxa are examined. The other mitochondrial markers, Cytb and CO3, are less often used in insect systematics. In sucking lice, these markers exhibit high levels of sequence divergence (Table 4) and phylogenies built using Cytb and CO3 lack support at deeper nodes (Fig. 2b and c). Furthermore, due to the fast-evolving nature of these markers, it was difficult to find and design conserved primers and we were therefore unable to amplify Cytb and CO3 for several louse taxa (including the outgroup taxa; Table 2). Although Cytb and CO3 may have limited phylogenetic utility at higher taxonomic levels in lice, they could serve as useful markers for within-species studies.

Of the nuclear markers, 18S rRNA shows the lowest average pairwise sequence divergence (Table 4) but nevertheless produced a tree topology that was well supported (Fig. 2d). This marker has traditionally been useful in resolving higher-level taxonomic questions in lice and other insects (e.g., Whiting, 2002; Barker et al., 2003) and, as further demonstrated by the results of this study, 18S rRNA remains an important marker for louse phylogenetics. Length variation, however, is fairly common in 18S rRNA (Xie et al., 2008), making this marker difficult to align, even when using a reliable reference sequence for secondary structure to align closely related taxa. Smaller fragments, such as those identified by Barker et al. (2003), may be easier to work with and may be equally informative taxonomically. The other five nuclear markers (EF-1α, CAD, Wg, Pol II, and H3) appear to have potential for future louse research (Table 4 and Fig. 2). Among the four families of lice examined in this study, these markers showed intermediate levels of sequence divergence and may therefore be useful for studies among louse orders. As stated above, EF-1α is already commonly used and we expect that it will remain a valuable marker for louse systematics. We were able to successfully amplify and sequence the remaining four markers (CAD, Wg, Pol II, and H3) in the majority of our taxa using general insect primers (Table 2), although it was necessary to design more species-specific primers for Pol II. Furthermore, phylogenetic analysis of these four markers, with one exception (see below), resulted in trees that were topologically similar when the markers were examined individually or combined (Figs. 2 and 3). Continued analysis of these markers with increased taxon sampling will further elucidate their utility within Anoplura as well as other louse groups.

Only H3 exhibited a significantly different gene genealogy compared to the other eight markers (Fig. 2i), but there was no overall effect on topology or support values when H3 was included in a combined phylogenetic analysis of all molecular markers (Fig. 3). It is likely that the overwhelming signal from the other markers in the combined analysis masked the contradictory signal from H3. In general, when multiple molecular markers are being used to infer a species tree, it may be preferable to utilize methodologies that are able to simultaneously estimate gene and species trees while allowing for independent evolutionary processes for each locus (e.g., Bayesian Estimation of Species Trees or BEST; Edwards et al., 2007; Liu and Pearl, 2007) rather than relying on an analysis that concatenates the data. This type of approach also is useful if, due to short branch lengths in the species tree, gene trees that do not match the species tree are more common than gene trees matching the species tree, thus resulting in incorrect inferences of species relationships (anomalous gene trees; Degnan and Rosenberg, 2006; Leaché and McGuire, 2006; Edwards et al., 2007; Kubatko and Degnan, 2007). Unfortunately, we were unable to use BEST in this study because we had incomplete data sets (Table 2) for nearly every marker we examined. However, given that all other molecular markers resulted in similar and non-contradictory phylogenies when analyzed independently (Fig. 2), the tree in Fig. 3 most likely represents the species phylogeny. Thus, the gene tree produced by H3 does not appear to represent a reliable estimate of species relationships and it is possible that, due to the age of lice examined in this study, evolutionary processes such as hybridization or gene duplication and extinction are the cause of this disparate gene genealogy (Maddison, 1997). Taken together, analysis of multiple genes in a combined framework offer an advantage over single-marker studies by providing a robust assessment of phylogenetic relationships and an opportunity to better understand evolutionary processes operating among genes and taxa (Wiens, 2006; Ceotto et al., 2008; Hughes et al., 2007).

4.2. Estimates of divergence times

A second goal of this study was to employ multiple molecular markers, calibration points, and methodologies to estimate divergence times among primate louse lineages. Several studies have advocated the use of multiple calibration points to obtain the most accurate estimates of divergence times (Wiegmann et al., 2003; Yang and Yoder, 2003; Yoder and Yang, 2004; Near et al., 2005; Won and Renner, 2006; Yang and Rannala, 2006; Hug and Roger, 2007; Manos et al., 2007; Rutschmann et al., 2007), especially when estimating recent divergence events (Douzery et al., 2003) and when calibrations are basally and terminally located (Porter et al., 2005). The findings reported here support these recommendations. Analysis of individual genes yielded highly variable results, especially when only one calibration point was used and when estimates were made for nodes located farther away from the calibration point (Appendices A and B). Additionally, the use of only the basal calibration point resulted in overestimates of all terminal nodes whereas the use of only the terminal calibration point resulted in underestimates of more basal nodes. Even nodes of known cospeciation (Nodes 1 and 4; Reed et al., 2004, 2007) were overestimated or underestimated when the alternative calibration point was used. However, when both terminal and basal calibrations were employed, divergence estimates tended to be less variable among genes although confidence intervals were generally larger for nuclear than for mitochondrial genes.

Because the estimation of divergence times from single markers can be problematic (see above; Edwards and Beerli, 2000; Arbogast et al., 2002; Thorne and Kishino, 2002), we concentrate our discussion on the analyses of combined data sets. The use of multiple markers resulted in divergence estimates that were much less variable than single-locus estimates (Table 5). Although Bayes factors support partitioning the data, divergence estimates in BEAST did not differ noticeably among partitioned and non-partitioned data sets, or with the *multidivtime* analyses (Table 5). Estimates of divergence times of the combined data set support divergence times of roughly 3 and 13 Ma between the two *Pthirus* species (*Pthirus gorillae* and *Pthirus pubis*; Fig. 4, Node 3) and between *Pediculus* and *Pthirus* (Fig. 4, Node 2), respectively (Table 5). Furthermore, the *multidivtime* 95% credibility intervals and the BEAST 95% HPD for these estimates do not include the divergence time of the hosts (7–9 Ma for humans and gorillas; Table 5). Thus, we support the findings from Reed et al. (2007) and infer both a host switch of *Pthirus* from gorillas to humans and an ancient louse speciation event on the common ancestor of these great apes.

In this study, we also estimated divergences within *Pediculus humanus*, specifically the divergence of lice belonging to the mitochondrial Clade A (Node 6, head and body lice) and Clade B (Node 7, head lice only; Figs. 1 and 4) defined by Reed et al. (2004). In agreement with several other studies, the divergence of *Pediculus humanus* (Node 5) occurred 1–2 million years ago (Table 5; Kittler et al., 2003, 2004; Reed et al., 2004; Light et al., 2008) and is significantly older than the origin of modern humans (roughly 100,000 years ago). Additionally, within *Pediculus humanus*, lice belonging

to the mitochondrial Clade A (Node 6) are substantially older than lice belonging to Clade B (this difference is more apparent with our BEAST estimates; Table 5). Using mitochondrial markers, Reed et al. (2004) determined that the divergence of lice belonging to Clade A was roughly 540,000 years whereas the divergence of Clade B was 150,000 years. Our estimates using both mitochondrial and nuclear markers of 300,000 and 90,000 years (for partitioned BEAST analyses: Table 5) are slightly younger and less variable than those of Reed et al. (2004). However, these estimates are very recent and additional population-level analyses are necessary to produce more reliable estimates of divergences within *Pediculus humanus*. It is still clear, however, that these two louse clades have likely experienced drastically different evolutionary histories and that future research is necessary to explain the ancient origin of *Pediculus humanus* on humans.

We employed two Bayesian approaches, *multidivtime* (Kishino et al. 2001; Thorne and Kishino 2002) and BEAST (Drummond and Rambaut 2007), to estimate divergence times in this study. Both of these analytical procedures are similar in that they relax the molecular clock (allow rates to vary across the tree) and allow for rate variation among genes rather than combining the information into a single data set (i.e., r8s; Sanderson, 2003). To estimate divergence times, *multidivtime* requires a user-defined tree whereas BEAST can simultaneously estimate the tree topology as it estimates divergence times (Drummond et al., 2006). In our BEAST analyses, however, monophyly was enforced for all nodes of interest to conform to the best tree (Fig. 3), thus we essentially used a user-defined tree just as in *multidivtime*. Analyses allowing estimation of both the tree topology and divergence times of the combined 9- and 8-gene data sets resulted in estimates that were nearly identical to those presented in Table 5 (data available upon request). Therefore, topological constraints within BEAST had no effect on estimates of divergence time. BEAST also differs from other methodologies because it allows for uncertainty in calibration points. Dating programs such as *multidivtime* and r8s require that calibration points be fixed (i.e., hard bounds) thereby failing to fully account for imperfect calibration points (Yang and Rannala, 2006; Sanders and Lee, 2007). In the BEAST analyses performed here, calibrations were assumed to have a normal prior distribution. While other studies have found somewhat disparate estimates when using other prior distributions (e.g., uniform or log-normal; Leaché and Mulcahy, 2007; Sanders and Lee, 2007), we believe our estimates are a good representation of primate louse divergence times because BEAST analyses produced very similar estimates to *multidivtime* (Table 5) and r8s (data available upon request). We note, however, that *multidivtime* tended to produce more recent age estimates (especially for terminal nodes; Table 5). Therefore, we feel that both BEAST and *multidivtime*, given multiple calibrations as well as multiple molecular markers, were equally able to produce reliable estimates of divergence times.

Parasites that have cospeciated with their hosts track host history and data from parasites can potentially be used as independent markers to make inferences about host evolutionary history. Although our chosen 20–25 and 5–7 Ma calibrations points are commonly used in primate studies (Stauffer et al., 2001; MacLatchy, 2004; Young and MacLatchy, 2004; Steiper et al., 2004), there remains debate about primate divergence times (Kumar et al., 2005; Patterson et al., 2006; Suwa et al., 2007). Thus, additional studies of cospeciating taxa could provide valuable information to help resolve issues of primate phylogenetic relationships and divergences. Studies of parasite taxa also can uncover entirely new aspects of primate evolutionary history. For example, our estimates of louse divergence times further support a host switch, roughly 3 Ma, between archaic hominids and gorillas (Reed et al. 2007). This host switch suggests that 3 Ma archaic humans and gorillas were in some form of contact, possibly habitat sharing or predation, and it is likely that this event would not have been hypothesized using host data alone. Exactly how this host switch occurred and was successful, however, will require future research. Clearly there are still many unknowns regarding primate evolutionary history and data from cospeciating parasites and other associated taxa have the potential to fill in the gaps.

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Appendix A

Divergence times for primate louse clades based on *multidivtime* analysis of individual genes (except for Cytb, CO3, and H3; see text). Values shown are the average and 95% credibility intervals (in parentheses). See text and Fig. 1 for data set and Node definitions.

Data set	Node 1	Node 2	Node 3	Node 4	Node 5	Node 6	Node 7
Basal calibration (Node 1)[a]							
COI	22.56 (20.13, 24.86)	15.80 (10.58, 21.19)	6.72 (2.57, 13.34)	10.47 (4.93, 16.85)	4.30 (1.01, 10.73)	0.77 (0.04, 3.93)	0.38 (0.01, 2.31)
18S rRNA	22.47 (20.11, 24.87)	16.99 (9.80, 23.04)	2.36 (0.13, 8.17)	9.66 (3.85, 16.65)	5.94 (1.64, 12.60)	5.00 (1.20, 11.24)	3.18 (0.29, 9.02)
EF-1α	22.52 (20.11, 24.87)	16.17 (8.63, 22.92)	2.46 (0.07, 9.14)	9.03 (2.89, 17.21)	5.03 (0.98, 12.15)	3.60 (0.49, 9.81)	2.03 (0.06, 7.33)
CAD	22.72 (20.18, 24.89)	19.09 (13.19, 23.66)	2.46 (0.15, 8.54)	12.62 (6.01, 18.66)	4.98 (0.77, 11.46)	3.51 (0.40, 9.17)	2.33 (0.01, 6.79)
Pol II	22.62 (20.14, 24.87)	19.89 (13.95, 24.02)	2.42 (0.08, 9.94)	12.10 (4.84, 19.15)	4.65 (0.76, 11.59)	3.06 (0.33, 8.78)	2.16 (0.07, 7.54)
Wg	22.41 (20.12, 24.83)	17.98 (9.49, 23.65)	5.02 (0.27, 16.44)	12.93 (4.55, 21.17)	6.05 (0.98, 14.94)	3.96 (0.44, 11.42)	2.85 (0.10, 9.71)
Terminal calibration (Node 4)[b]							
COI	18.81 (10.89, 30.56)	11.24 (7.39, 16.63)	3.75 (1.70, 7.30)	5.99 (5.05, 6.94)	1.67 (0.83, 3.35)	0.15 (0.02, 0.53)	0.09 (0.00, 0.31)
18S rRNA	18.68 (9.78, 35.75)	13.03 (7.07, 23.91)	1.71 (0.09, 6.10)	6.00 (5.05, 6.94)	3.56 (1.31, 6.15)	2.96 (0.88, 5.50)	1.89 (0.19, 4.63)
EF-1α	22.92 (9.27, 48.02)	14.28 (6.90, 28.29)	1.85 (0.05, 7.91)	6.00 (5.05, 6.95)	3.15 (0.91, 5.85)	2.25 (0.44, 4.79)	1.21 (0.04, 3.65)
CAD	18.79 (9.75, 35.37)	13.74 (7.65, 24.77)	1.12 (0.08, 4.82)	6.10 (5.07, 6.96)	1.60 (0.33, 3.87)	1.12 (0.16, 3.03)	0.71 (0.03, 2.34)
Pol II	17.80 (8.47, 35.48)	14.60 (6.97, 28.65)	1.37 (0.47, 5.63)	6.03 (5.05, 6.95)	1.90 (0.43, 4.72)	1.24 (0.18, 3.50)	0.87 (0.04, 2.96)
Wg	13.21 (6.31, 30.93)	9.73 (5.56, 20.86)	2.71 (0.13, 8.56)	5.97 (5.04, 6.94)	2.63 (0.56, 5.74)	1.71 (0.24, 4.51)	1.20 (0.04, 3.90)
Basal and terminal calibrations							
COI	22.10 (20.10, 24.78)	12.62 (9.59, 16.06)	4.16 (2.17, 7.68)	6.12 (5.08, 6.96)	1.58 (0.84, 2.97)	0.13 (0.02, 0.40)	0.08 (0.00, 0.25)
18S rRNA	22.20 (20.10, 24.81)	14.93 (8.84, 21.36)	2.00 (0.10, 6.68)	6.09 (5.07, 6.96)	3.70 (1.42, 6.17)	3.06 (0.99, 5.53)	1.97 (0.22, 4.68)
EF-1α	22.33 (20.11, 24.85)	14.33 (8.12, 21.26)	1.92 (0.06, 7.25)	6.05 (5.06, 6.95)	3.22 (0.97, 5.95)	2.33 (0.45, 4.99)	1.25 (0.05, 3.82)
CAD	22.16 (20.09, 24.79)	15.74 (9.85, 21.60)	1.33 (0.08, 5.48)	6.18 (5.10, 6.97)	1.61 (0.33, 4.12)	1.13 (0.17, 3.23)	0.72 (0.03, 2.49)
Pol II	22.16 (20.08, 24.80)	17.92 (11.41, 23.08)	1.64 (0.04, 6.46)	6.13 (5.08, 6.96)	1.91 (0.42, 5.00)	1.25 (0.17, 3.69)	0.87 (0.03, 2.96)
Wg	22.11 (20.09, 24.81)	14.58 (6.98, 22.33)	3.74 (0.19, 12.11)	6.11 (5.08, 6.96)	2.80 (0.59, 5.94)	1.84 (0.24, 4.79)	1.29 (0.05, 4.12)

[a] Basal calibration of 20–25 Ma at Node 1 corresponding to the split between old world monkeys and apes.
[b] Terminal calibration of 5–7 Ma at Node 4 corresponding to the split between chimpanzees and humans.

Appendix B

Divergence times for primate louse clades based on a relaxed phylogenetic analysis (BEAST) of analysis of individual genes (except for Cytb, CO3, and H3; see text). Values shown are the posterior mean ages and 95% HPD (in parentheses). See text and Fig. 1 for data set and Node definitions.

Data set	Node 1	Node 2	Node 3	Node 4	Node 5	Node 6	Node 7
Basal calibration (Node 1)[a]							
COI	22.29 (19.35, 25.23)	13.70 (10.35, 17.36)	4.51 (2.68, 6.48)	6.84 (4.49, 9.40)	1.76 (0.97, 2.65)	0.12 (0.03, 0.24)	0.08 (0.00, 0.19)
18S rRNA	22.29 (19.33, 25.20)	14.57 (7.58, 21.75)	3.58 (0.07, 10.11)	9.58 (3.23, 16.78)	5.59 (1.31, 11.30)	4.55 (1.01, 9.38)	3.44 (0.15, 7.91)
EF-1α	22.29 (19.35, 25.24)	13.06 (6.67, 20.93)	1.82 (0.00, 7.90)	6.58 (1.69, 15.21)	3.28 (0.38, 10.52)	2.35 (0.20, 7.24)	0.98 (0.00, 4.20)
CAD	22.35 (19.41, 25.33)	18.28 (10.46, 23.99)	10.62 (1.42, 19.68)	14.48 (6.63, 22.02)	10.29 (3.41, 18.15)	7.52 (1.40, 14.36)	4.01 (0.00, 10.97)
Pol II	22.32 (19.42, 25.31)	17.00 (8.95, 23.72)	7.70 (0.00, 18.67)	12.67 (3.50, 21.36)	8.52 (0.25, 17.69)	6.00 (0.15, 13.94)	3.34 (0.00, 10.84)
Wg	22.35 (19.44, 25.37)	18.38 (10.13, 24.34)	7.41 (0.00, 17.43)	14.37 (6.07, 21.80)	10.52 (3.29, 18.23)	7.34 (1.30, 14.46)	4.19 (0.00, 11.43)
Terminal calibration (Node 4)[c]							
COI	19.33 (12.41, 26.67)	11.87 (8.05, 15.91)	3.90 (2.20, 5.75)	5.91 (4.94, 6.90)	1.51 (0.84, 2.25)	0.11 (0.02, 0.21)	0.07 (0.01, 0.16)
18S rRNA	13.79 (6.07, 25.08)	9.02 (5.10, 15.57)	2.22 (0.04, 5.67)	5.92 (4.91, 6.88)	3.47 (1.35, 5.72)	2.83 (0.82, 4.95)	2.15 (0.11, 4.42)
EF-1α	21.03 (6.15, 41.61)	12.11 (5.38, 21.71)	1.46 (0.00, 5.05)	5.91 (4.95, 6.93)	2.75 (0.70, 5.33)	1.97 (0.34, 4.20)	0.97 (0.00, 2.68)
CAD	9.53 (5.34, 16.27)	7.80 (4.89, 12.51)	3.64 (0.01, 7.60)	5.94 (4.95, 6.92)	4.15 (1.72, 6.37)	3.02 (0.77, 5.40)	1.59 (0.00, 4.28)
Pol II	10.99 (5.14, 23.82)	8.30 (4.74, 17.99)	3.33 (0.00, 7.31)	5.92 (4.91, 6.89)	3.84 (0.54, 6.28)	2.70 (0.24, 5.27)	1.48 (0.00, 4.35)
Wg	10.31 (5.40, 18.98)	8.24 (4.92, 14.16)	3.45 (0.00, 8.56)	5.94 (4.94, 6.91)	4.28 (1.80, 6.42)	2.97 (0.64, 5.36)	1.71 (0.00, 4.47)
Basal and terminal calibrations							
COI	21.94 (19.22, 24.71)	13.09 (10.34, 16.06)	4.26 (2.74, 5.83)	6.11 (5.24, 6.98)	1.61 (1.03, 2.27)	0.11 (0.03, 0.21)	0.08 (0.00, 0.17)
18S rRNA	22.06 (19.09, 24.94)	12.84 (6.24, 19.15)	2.65 (0.04, 7.19)	6.06 (5.13, 7.02)	3.82 (1.92, 5.69)	3.22 (1.44, 4.95)	2.43 (0.24, 4.61)
EF-1α	22.41 (19.50, 25.35)	12.77 (7.71, 18.23)	1.23 (0.00, 4.28)	5.91 (4.94, 6.88)	2.52 (0.69, 4.92)	1.88 (0.42, 3.86)	0.65 (0.00, 2.26)
CAD	21.95 (19.02, 24.96)	13.96 (6.06, 22.09)	7.19 (0.02, 16.32)	6.11 (5.15, 7.10)	4.86 (2.18, 6.79)	3.69 (1.10, 6.01)	1.96 (0.00, 4.96)
Pol II	22.29 (19.32, 25.14)	16.89 (8.34, 23.07)	1.38 (0.00, 5.81)	5.96 (5.01, 6.95)	1.59 (0.23, 5.27)	1.14 (0.12, 3.79)	0.54 (0.00, 2.18)
Wg	22.01 (19.10, 25.01)	13.68 (5.69, 22.04)	6.40 (0.00, 16.02)	6.10 (5.15, 7.06)	4.93 (2.41, 6.87)	3.59 (1.01, 6.00)	2.10 (0.00, 5.04)

[a] Basal calibration of 20–25 Ma at Node 1 corresponding to the split between old world monkeys and apes.
[b] 9-gene and 8-gene data sets were partitioned in BEAST.
[c] Terminal calibration of 5–7 Ma at Node 4 corresponding to the split between chimpanzees and humans.

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