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Authors

Joseph, Sandeep J
Marti, Hanna
Didelot, Xavier
et al.

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Chlamydiaceae Genomics Reveals Interspecies Admixture and the Recent Evolution of *Chlamydia abortus* Infecting Lower Mammalian Species and Humans

Sandeep J. Joseph¹, Hanna Marti², Xavier Didelot³, Santiago Castillo-Ramirez⁴, Timothy D. Read^{1,5,†}, and Deborah Dean^{2,6,7,*}

¹Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine

²Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, Oakland, California

³Department of Infectious Disease Epidemiology, Imperial College, London, United Kingdom

⁴Programa de Genomica Evolutiva, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

⁵Department of Human Genetics, Emory University School of Medicine

⁶Department of Medicine, University of California, San Francisco

⁷Joint Graduate Program in Bioengineering, University of California, San Francisco, and University of California, Berkeley

*Corresponding author: E-mail: ddean@chori.org.

†These authors contributed equally to this work.

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Abstract

Chlamydiaceae are obligate intracellular bacteria that cause a diversity of severe infections among humans and livestock on a global scale. Identification of new species since 1989 and emergence of zoonotic infections, including abortion in women, underscore the need for genome sequencing of multiple strains of each species to advance our knowledge of evolutionary dynamics across *Chlamydiaceae*. Here, we genome sequenced isolates from avian, lower mammalian and human hosts. Based on core gene phylogeny, five isolates previously classified as *Chlamydia abortus* were identified as members of *Chlamydia psittaci* and *Chlamydia pecorum*. *Chlamydia abortus* is the most recently emerged species and is a highly monomorphic group that lacks the conserved virulence-associated plasmid. Low-level recombination and evidence for adaptation to the placenta echo evolutionary processes seen in recently emerged, highly virulent niche-restricted pathogens, such as *Bacillus anthracis*. In contrast, gene flow occurred within *C. psittaci* and other *Chlamydiaceae* species. The *C. psittaci* strain RTH, isolated from a red-tailed hawk (*Buteo jamaicensis*), is an outlying strain with admixture of *C. abortus*, *C. psittaci*, and its own population markers. An average nucleotide identity of less than 94% compared with other *Chlamydiaceae* species suggests that RTH belongs to a new species intermediary between *C. psittaci* and *C. abortus*. Hawks, as scavengers and predators, have extensive opportunities to acquire multiple species in their intestinal tract. This could facilitate transformation and homologous recombination with the potential for new species emergence. Our findings indicate that incubator hosts such as birds-of-prey likely promote *Chlamydiaceae* evolution resulting in novel pathogenic lineages.

Key words: *Chlamydiaceae*, comparative genomics, homologous recombination, molecular clock, population structure, red-tailed hawk.

Introduction

Chlamydiaceae are a family of obligate intracellular bacteria with nine species. Until 1992, there were only four recognized species of the genus *Chlamydia*: *Chlamydia trachomatis* and

Chlamydia psittaci were established in 1968 followed by *C. pneumoniae* in 1989 and *Chlamydia pecorum* in 1992 (Fukushi and Hirai 1992). The remaining five species were identified in 1999 (Everett et al. 1999). In 1999, because of

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shared morphological similarities, *Chlamydiaceae* species were reclassified into two separate genera, *Chlamydia* and *Chlamydophila* (Everett et al. 1999). Despite the differences in chromosome size (~1.05 Mb) for the *Chlamydia* species *C. trachomatis*, *C. muridarum* and *Chlamydia suis* compared with the six species of the *Chlamydophila* lineage (~1.2 Mb), there was insufficient chromosomal evidence for two separate genera, and they have now been reunited under the genus *Chlamydia* (Greub 2010; Sachse et al. 2015). In 2014, two new species were accepted: *Chlamydia avium* infects pigeons and psittacine birds, whereas *Chlamydia gallinacea* infects chickens, guinea fowl and turkeys (Sachse et al. 2014).

Chlamydia trachomatis exclusively infects humans and remains the leading cause of bacterial sexually transmitted diseases and preventable blindness worldwide (Dean et al. 2013; Shao et al. 2013). Although *C. pneumoniae* originated from zoonotic transmission, it is primarily a human pathogen responsible for endemic infection as well as epidemic outbreaks of respiratory and ocular disease (Bodetti et al. 2002; Myers et al. 2009; Roulis et al. 2013). *Chlamydia psittaci* is well recognized for its zoonotic potential, causing psittacosis, an upper respiratory infection that can be life-threatening if it becomes systemic, as well as eye diseases in humans. It also causes disease in wild birds and domesticated animals (Longbottom and Coulter 2003; Harkinezhad et al. 2009; Reinhold et al. 2011). Although *Chlamydia abortus* is mainly responsible for ovine enzootic abortion, it has emerged as a dangerous pathogen for pregnant women (Longbottom and Coulter 2003). *Chlamydia pecorum* and *C. suis* are important livestock pathogens (Mohamad and Rodolakis 2010; Reinhold et al. 2011; Schautteet and Vanrompay 2011), whereas *Chlamydia felis*, *Chlamydia muridarum*, and *Chlamydia caviae* infect cats, rodents, and guinea pigs, respectively.

The ongoing discovery of new *Chlamydiaceae* species and strains infecting novel animal hosts, such as frogs and koalas, and the recent knowledge of human abortions induced by zoonotic *Chlamydia* species (Bodetti et al. 2002; Joseph et al. 2011; Somboonna et al. 2011; Sachse et al. 2014) indicate an urgent need to more fully understand *Chlamydiaceae* evolution. Comparative genomics projects of *C. trachomatis* using dozens of strains (Harris et al. 2012; Joseph et al. 2012) have revealed much about pathogen population structure, including clade specificity for disease phenotypes and relatively frequent recombination without evidence for gene gain by horizontal gene transfer and little DNA sequence loss by deletion (Joseph et al. 2011, 2012; Joseph and Read 2012).

In contrast, progress in comparative genomics of other *Chlamydiaceae* has been slow, largely because of technical difficulties in isolating and culturing strains from each species (Read et al. 2013; Bachmann, Polkinghorne, et al. 2014). A study of 20 *C. psittaci* genomes discovered frequent switching of DNA among strains from different avian and mammalian hosts, and homologous recombination that occurred more often than for *C. trachomatis* (Read et al. 2013). This study

also revealed a clonal epidemic expansion of the species in North America and the recent emergence of the disease psittacosis originating from New World parrots. A comparative study of four *C. pecorum* genomes identified limited variations between strains, but found differences in the number of pseudogenes (Sait et al. 2014), whereas a study that included these four strains and four additional ones concluded that variation is largely determined by single nucleotide polymorphisms (SNPs) (Bachmann, Fraser, et al. 2014). Another study identified mixed populations of genetically distinct strains of *C. pecorum* obtained from koalas and sheep (Bachmann et al. 2015). Aside from these studies, only a few genomes are available that cover other *Chlamydiaceae* species: Two for *C. abortus* (Thomson et al. 2005); and one each for *C. felis* (Azuma et al. 2006), *C. suis* (Donati et al. 2014), *C. muridarum* (Read et al. 2000), and *C. caviae* (Read et al. 2003).

Despite the plethora of human and nonhuman diseases that exact a huge economic burden globally, there have been no comparative genomic studies of the different *Chlamydiaceae* species to date. The fact that similar diseases are caused by the different species and that many species infect a diversity of hosts (supplementary table S1, Supplementary Material online) suggests that genomics, at least in part, could explain host–pathogen interactions and disease. To better understand their interactions and evolutionary processes, we analyzed the genomes of 36 isolates from human, lower mammalian, and avian species.

Materials and Methods

Chlamydiaceae Species/Strains, Clonal Purification, and Generation of Genomic DNA

In this study, we sequenced five *C. abortus*, three *C. pecorum*, and two *C. psittaci* strains (supplementary table S2, Supplementary Material online). In addition, we included the published whole-genome sequencings (WGSs) of 31 other *Chlamydiaceae* strains, including those from *C. pneumoniae*, *Chlamydia avium*, *C. felis*, *C. caviae*, *C. gallinacea*, *C. trachomatis*, and *C. muridarum* (supplementary table S2, Supplementary Material online). Although the majority of the strains were of lower mammalian and avian origin, six strains were isolated from humans. Each of the ten new strains was individually propagated in McCoy or HeLa 229 cells, clonally purified, and treated with DNase prior to gDNA purification as previously described (Somboonna et al. 2011; Joseph et al. 2012; Read et al. 2013).

Genome Sequencing

Genomes were sequenced using GS-FLX (454 Life Sequencing Inc., Branford, CT). Libraries for sequencing were prepared from 1 to 5 µg of genomic DNA. De novo assembly was performed using Newbler (Knight J, personal communication) and default parameters. Further processing of the genomes

was performed as previously described (Joseph et al. 2012). The contigs from *C. abortus*, *C. pecorum* and *C. psittaci* were aligned against the previously sequenced *C. abortus* S26/3, *C. pecorum* E58 and *C. psittaci* 6BC genomes, respectively, using abacus (<http://abacas.sourceforge.net/Manual.html>, last accessed April, 2015) to create concatenated, ordered “pseudocontigs.” These pseudocontigs were considered as the bacterial chromosome for each strain and annotated using the Prokka bacterial genome annotation pipeline (Seemann 2014). For comparative analysis, all published genomes used in this study were also re-annotated using the same pipeline. Pairwise comparison of genomes was conducted by calculating average nucleotide identity (ANI) as described previously (Richter and Rosselló-Móra 2009) using JSpecies (www.imedea.uib.es/jspecies, last accessed June, 2015) with default parameters. The raw genome data generated for this study are deposited in the SRA database under the following study accession numbers: SRP060457 (EP1), SRP060463 (EP6), SRP060476 (EBA), SRP060475 (AC1), SRP060473 (EAE-LX), SRP060471 (B577), SRP060468 (24-26), SRP060466 (OSP), SRP060465 (JP394), and SRP060464 (757).

Whole-Genome Alignment and Identification of Core Genes

Three whole-genome MAUVE (Darling et al. 2004, 2010) progressive alignments were computed. The first consisted of 36 genomes from *C. psittaci*, *C. abortus*, *C. pecorum*, *C. pneumoniae*, *C. caviae*, *C. felis*, *C. avium* and *C. gallinacea*, which was used for ClonalFrame (Didelot et al. 2010), fineSTRUCTURE (Yahara et al. 2013), BAPS (Tang et al. 2009) and phylogenetic analyses (see below). In the second alignment, we added four *C. trachomatis* genomes from each of the four *C. trachomatis* clades and a single *C. muridarum* genome (total of 41 strains). The third alignment consisted of 32 genomes sans *C. caviae*, *C. felis*, *C. avium*, and *C. gallinacea* for a separate ClonalFrame analysis. The core Locally Collinear Block (LCB) from the second MAUVE alignment was extracted and concatenated to form a super alignment for use in phylogenetic analyses described below.

The complete predicted proteome from all 36 annotated/reannotated genomes was searched against itself using BLASTP with an *e*-value cutoff of $1e-05$. The best BLASTP scores were utilized for identifying orthologous sequences using the OrthoMCL algorithm as described (Li et al. 2003); the pan-matrix containing all information regarding orthologous genes was generated. This pan-matrix was imported into the R-package called micropan (Snipen and Liland 2015) to generate the visualizations describing the *Chlamydiaceae* pan genome. Core genes are defined as the protein-coding gene clusters that are shared by all *Chlamydiaceae* strains. Unique genes found in only one strain were also identified. MUSCLE (Edgar 2004) was used with default settings to align core genes;

each protein alignment was filtered by GBLOCKS (Castresana 2000) to remove gaps and highly divergent regions.

Phylogenetic Reconstruction

The core LCBs extracted from the second MAUVE alignment of 41 strains (see above) were concatenated for phylogenetic analysis. We also concatenated alignments of all core protein families and reconstructed the phylogeny. The maximum-likelihood (ML)-based phylogenetic reconstruction was implemented using RAxML (Stamatakis et al. 2012). For whole-genome alignment and core protein sequence alignment, the branch lengths/evolutionary distances were estimated, respectively, using the GTR (general time reversible) nucleotide substitution model and Jones–Taylor–Thornton (JTT) amino acid substitution model of rate heterogeneity with four discrete rate categories. To evaluate statistical support, a majority rule-consensus tree of 100 bootstrap replicates was computed. We also used the core of the first and third MAUVE alignments (36 and 32 genomes, respectively) to reconstruct phylogenies using the coalescent-based ClonalFrame method (Didelot et al. 2010) (see below). For the phylogeny of the 17 plasmids, we performed OrthoMCL clustering of all genes, identifying five complete genes found in all 17 plasmids that were concatenated and aligned using progressiveMAUVE. Three sets of ClonalFrame analysis were performed (see below). For the ML tree, we removed the recombinant regions identified by ClonalFrame and performed RAxML using the same parameters as for the chromosome (above).

Analysis of Homologous Recombination

ClonalFrame (version 1.2) (Didelot et al. 2010) was run for 40,000 iterations on the whole-genome core nucleotide alignment identified in the first (36 genomes) and third (32 genomes) MAUVE alignments; the initial half was discarded as Markov chain Monte Carlo (MCMC) burn-in. Three independent and parallel runs of ClonalFrame were performed. Their reconstructed phylogenies and recombination events across the three runs had high congruence. Additionally, for each reconstructed branch substitution event introduced by either mutation or recombination, the number of mutation and recombination events were computed. The relative effect of recombination and mutation on genetic change (r/m) and the relative rate of mutation and recombination (ρ/θ) were estimated as described (Joseph et al. 2012; Read et al. 2013).

Molecular Clock Analysis

Bayesian analysis of evolutionary rates and divergence times was performed based on the MAUVE alignment using BEAST v1.8.2 with the HKY (Hasegawa–Kishino–Yano) substitution model and tip dates defined as year of isolation. We implemented two molecular clock models: Strict molecular clock with a constant size coalescent model, and the relaxed

molecular clock model along with the Bayesian skyline demographic model. Three independent runs of BEAST for both models, each with 300 million MCMC iterations, sampling every 10,000 iterations with the first 10% discarded as burn-in, were performed. These three runs were combined to provide robust estimates of posterior parameter distributions (calculated using Tracer v1.5).

ClonalFrame also estimates the clonal genealogy as well as the number of mutations that occurred on each branch of the tree. Combining this information with isolation date for each genome (supplementary table S2, Supplementary Material online) allowed the simultaneous estimation of mutation rate per year and dates of existence of nodes in the ClonalFrame tree. This inference was performed assuming the Kingman coalescent with the temporally offset leaves model (Drummond et al. 2002) for the tree and a uniform prior for mutation rate.

Population Structure Analysis

To elucidate the possible population structure of the 36 *Chlamydomphila* lineage strains, the ChromoPainter algorithm was applied to the genome-wide haplotype data using the linkage model. A recombination map file was created by specifying a uniform recombination rate per-site per-generation using a Perl script called makeuniformrecfile.pl (<http://www.paintmychromosomes.com>). The output from ChromoPainter is a coancestry matrix that summarized the blockwise homology between the 36 genomes. The fineSTRUCTURE algorithm (Yahara et al. 2013) used the coancestry matrix generated by ChromoPainter to perform model-based clustering using a Bayesian MCMC approach to explore the population structure. FineSTRUCTURE was run for 400,000 iterations; the first 200,000 iterations were discarded as MCMC burn-in. The thinning interval was specified at 100.

To gain further insights into the population structure of these species, we used the BAPS software (Corander and Marttinen 2006) to identify genetically differentiated groups and determine the amount of admixture among these groups. We ran the BAPS clustering model with hierarchical manner to find substructures inside the main clusters (Cheng et al. 2013). To find optimal clustering, we ran five independent iterations with the prior upper bound of the number of clusters set to 35. The clustering was performed with four levels in the hierarchy. The first level gave 10 clusters, whereas the fourth yielded 20 clusters. We also conducted an admixture analysis based on the 20 clusters, considering the minimum number of individuals for a population as one and, other than that, we employed the same values that were used by Castillo-Ramirez et al. (2012).

Attribution of Origins to the Recombination Events

For each branch of the tree reconstructed by ClonalFrame, we defined recombined fragments as genomic intervals with a posterior probability of recombination above 0.50 at every

site, reaching 0.95 in at least one site, and a length of at least 100 bp (Didelot et al. 2011). Each such recombined fragment was searched for using BLAST (Basic Local Alignment Search Tool) against the whole database containing all “finished” genomes and plasmid sequences of *Chlamydiaceae* species minus strains of the clade affected by the import. The hits with the highest normalized BLASTN score along with a percent identity of at least 95% were kept. If these hits were with strains belonging to the same species, the origin of the event was attributed to ancestral nodes and called “ambiguous,” meaning imported from an unknown external source.

Substitution Rate (dN/dS) Calculations

Nonsynonymous (dN) and synonymous (dS) substitutions and dN/dS ratios were calculated, and protein alignments along with corresponding nucleotide sequences were converted to codon alignments using PAL2NAL (Suyama et al. 2006) as described (Joseph et al. 2012).

Results

The C. abortus Species Is a Recent Clonal Expansion within the *Chlamydomphila* Lineage

We applied 454 technology for WGS of ten previously unsequenced *Chlamydiaceae* strains of human and lower mammalian origin, originally typed by serology and disease etiology as *C. abortus* (supplementary table S2, Supplementary Material online). The ML tree created from an alignment of 36 *Chlamydomphila* genomes (10 from this study+26) plus 4 *C. trachomatis* and 1 *C. muridarum* (fig. 1) determined that two strains needed to be reclassified as *C. psittaci* and three as *C. pecorum*. Four of the true *C. abortus* strains (B577, EBA, AC1, and EAE/LX) caused abortion in sheep and cows, and one (24-26) caused pneumonia in sheep. The three newly sequenced *C. pecorum* strains (OSP, JP394, and 757) were isolated from sheep abortion specimens whereas the two new *C. psittaci* strains, EP6 and EPI, caused abortion in a human and pneumonia in a sheep, respectively. None of the *C. abortus* strains contained the typical 7.5-kb *Chlamydiaceae* plasmid, whereas all *C. psittaci* and *C. pecorum* strains used in this study had the plasmid. However, none of the previously published *C. pecorum* genomes reported the presence of a plasmid. In the plasmid phylogeny (supplementary fig. S1, Supplementary Material online), each species is separated into monophyletic clades. Surprisingly, the plasmids of *C. pneumoniae* and *C. pecorum* shared a more recent ancestor with the *Chlamydia* species *C. trachomatis* and *C. muridarum* than with other *Chlamydomphila* species.

Including this study, there are now seven *C. abortus* genomes in the public domain. These genomes form a monomorphic group showing very little genetic variation (maximum ANI between two strains was 99.5%) (supplementary table

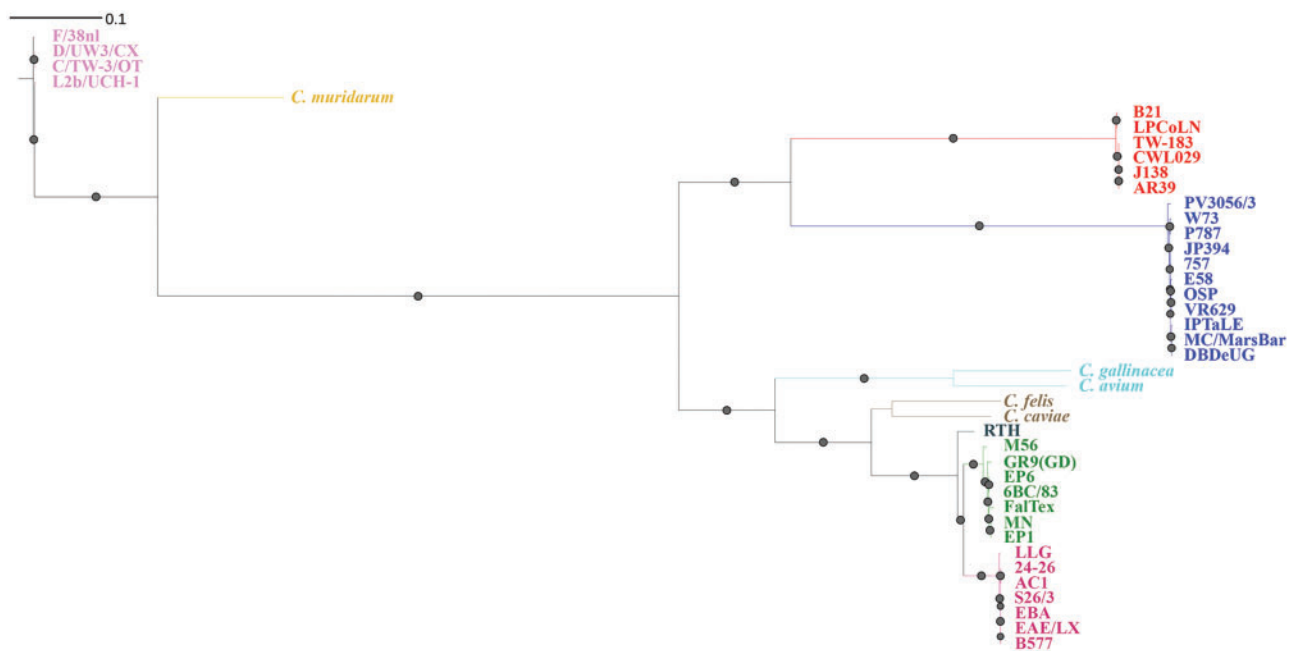


Fig. 1.—Whole-genome phylogeny of *Chlamydia* species and strains. The tree was constructed using ML approach utilizing RAXML based on the whole-genome alignment (see Materials and Methods). *Chlamydia pneumoniae* strains are highlighted in red, *C. pecorum* strains in blue, *C. psittaci* strains in green, *C. abortus* strains in magenta, and *C. trachomatis* in pink.

S3, Supplementary Material online) and very similar gene content (95% of genes are found in all seven strains). Interspecific genome comparisons show that the most variable portion of *Chlamydia* chromosomes for gene content is typically around the replication termination region termed the “plasticity zone” (PZ), although its boundaries are not consistently defined (Read et al. 2000; Thomson et al. 2005; Voigt et al. 2012; Sait et al. 2014). The ten strains sequenced in this study had PZ gene content similar to other members of their species (Voigt et al. 2012; Read et al. 2013; Sait et al. 2014).

To investigate further the variation in gene content within the *Chlamydomphila* lineage we created a genome set using the 10 genomes sequenced here plus 26 other published genomes consisting of *C. psittaci*, *C. pneumoniae*, *C. pecorum*, *C. avium*, *C. gallinacea*, *C. felis*, and *C. caviae* strains (supplementary table S4, Supplementary Material online). The matrix contained 1,449 unique gene clusters/families (supplementary fig. S2, Supplementary Material online). To describe the pan genome, we fitted a binomial mixture model on the pan matrix using the binomixEstimate function on micropan R package; the optimum number of components to characterize the gene clusters was estimated to be 6 (supplementary fig. S2a, Supplementary Material online). Principal component analysis performed using the pan matrix clustered the genomes into groups that were highly congruent to the inferred whole-genome phylogenetic clades (supplementary fig. S2b, Supplementary Material online). Supplementary figure S2c and d, Supplementary Material online, shows the distribution

of gene families across the clade and the rarefaction of the e pan-genome, respectively. We identified 668 core genes present among all 36 *Chlamydiaceae* strains, which represented 48.19–74.80% of the total number of genes in each genome. This is similar to earlier estimates (Collingro et al. 2011), illustrating the parsimony of conserved function within *Chlamydiaceae*.

Seven genes, present in similarity-based clusters, were only found in *C. abortus*. Six were short hypothetical genes in the PZ. The other was the rapidly evolving *IncA* gene, which encodes the inclusion membrane protein A (*IncA*) protein (Rockey et al. 1995). *Chlamydia pecorum* had 25 unique gene clusters with 21 hypothetical protein-coding genes and genes coding for aminopeptidase 2, phospholipase D precursor, polymorphic membrane protein 13 (*Pmp 13*), and *IncA*. *Chlamydia psittaci* had only one unique gene cluster encoding a hypothetical protein shared by all *C. psittaci* strains. All genes of known function found in the five *C. abortus*, three *C. pecorum*, and two *C. psittaci* genomes sequenced in this study have previously been identified in their species. In *C. pneumoniae*, 80 species-specific protein-coding gene families were identified, of which the majority were hypothetical genes. We also identified unique protein-coding genes (genes that did not cluster with any other genes) for each of the 36 strains.

Comparative analysis of 17 plasmid sequences of all the available *Chlamydiaceae* species revealed the presence of five core genes in their plasmids, which were replicative

DNA helicase, site-specific tyrosine recombinase (XerC, phage integrase family protein), sporulation initiation inhibitor protein (*soj*), and two hypothetical protein-coding genes. The gene coding for proteins P-6/P-7 was not present in *C. avium* and *C. psittaci* M56 strains.

To investigate the type and strength of selection across the 36 strains, we calculated the dN and dS substitutions for each of the 36 combinations of pairwise core genes and assessed the dN/dS ratio of each strain (Rocha et al. 2006; Hershberg and Petrov 2010) (supplementary fig. S4, Supplementary Material online). The median dN/dS ratios given the accumulation of dS substitution for *C. abortus* strains were higher compared with all other species, a result also suggested by Voigt et al. (2012).

Phylogenetic Analysis Places the *C. psittaci* Red-Tailed Hawk Strain As a Potential New Species within the Chlamydiaceae Family

We inferred a phylogeny for the *Chlamydophila* clade, which accounted for the effects of recombination by using ClonalFrame (Didelot and Falush 2007), where branch lengths were estimated in coalescent units of time (figs. 2 and 3). We used a whole-genome alignment of the 36 genomes consisting of 549 homologous regions (LCBs) identified by MAUVE software (Darling et al. 2004). These regions represented 676,097 bp of the 1.2 Mb average genome size. Predicted recombination events occurred across the entire portion of the conserved core alignment, as was seen in *C. trachomatis* and *C. psittaci* alignments (Harris et al. 2012; Joseph and Read 2012; Read et al. 2013). ClonalFrame provided two output statistics of the recombination rate in the *Chlamydophila* lineage: p/θ represents the frequency of occurrence of recombination relative to mutation, whereas r/m shows the importance of recombination on genetic diversification relative to mutation (supplementary table S4, Supplementary Material online). In *C. abortus*, the recombination rate was significantly lower than the other species, whereas its closest relative *C. psittaci* had the highest rate in the genus. For plasmids, ClonalFrame analysis on the core alignment of 17 *Chlamydiaceae* strains revealed significant evidence of recombination (fig. 4) except for all *C. psittaci* strains including the red-tailed hawk (RTH) strain. The estimated r/m ratio for the plasmids was 1.18 (95% CI; 0.37–3.144) and 0.076 (95% CI; 0.015–0.186) for p/θ .

In the recombination-corrected ClonalFrame phylogeny (figs. 2 and 3), all species clustered monophyletically into clades except the *C. psittaci* RTH strain (figs. 1 and 2). The two recently reported species, *C. avium* and *C. gallinacea*, formed a distinct clade, as did *C. caviae* and *C. felis*. In the ML tree, RTH was an outgroup of both *C. abortus* and the main *C. psittaci* group. However, in the ClonalFrame tree, RTH was in the *C. psittaci* clade, as previously reported (Read et al. 2013). The difference in ML phylogeny was likely due to

extensive recombination with *C. abortus* found in RTH (see below). It is notable that the RTH had an ANI less than 94% with other *C. psittaci* or *C. abortus* members (supplementary table S3, Supplementary Material online), which is below the threshold for assignment of the strain to a new species.

There was a reasonable correlation ($r=0.4$) with date of sample and root-to-tip distance on the ClonalFrame phylogeny, which allowed a molecular clock rate to be estimated (supplementary fig. S3, Supplementary Material online). Based on ClonalFrame analysis that differentiates between recombination and mutation events separately, the average mutation rate in *Chlamydiaceae* was found to be high ($1.42\text{e-}5$ per site per year with a 95% credibility interval of $1.41\text{e-}5$ – $1.43\text{e-}5$). BEAST-estimated mutation rates were even higher: $2.4778\text{e-}4$ (95% HPD interval $3.374\text{e-}11$ – $1.4353\text{e-}7$) for the relaxed skyline model and $1.8576\text{e-}4$ (95% HPD interval $3.363\text{e-}12$, $1.3408\text{e-}7$) for the strict molecular clock with a constant size coalescent model, in line with our previous estimates of $1.683\text{e-}4$ for BEAST and $1.74\text{e-}5$ for ClonalFrame (Read et al. 2013). With this fast rate, the molecular clock based on ClonalFrame placed the root of the *Chlamydophila* lineage at only 5570 BC. The split at the common ancestor of *C. abortus* and *C. psittaci* to form the two new species was predicted at AD 1–500, the split between RTH and *C. psittaci* at AD 719, the modern *C. psittaci* lineage seems to have emerged in the past 200 years (AD 1678 as we noted previously [Read et al. 2013]), and the split between *C. psittaci* and *C. abortus* at AD 1881 (supplementary fig. S3, Supplementary Material online). In the absence of historical data or specimens, the dates were based solely on mutation rates derived from recent genome sequences and should therefore be treated with caution.

Population Structure of the *Chlamydophila* Lineage

As ClonalFrame revealed that recombination had been a major factor in *Chlamydiaceae* evolution, we investigated how genetic variation is apportioned across species using BAPS (fig. 5) and fineSTRUCTURE software (fig. 6). Both methods establish genetically differentiated groups and infer possible admixture occurring among those groups. We used BAPS to implement a hierarchical Bayesian model-clustering based on the core SNP alignment, which produced 20 clusters at the finest hierarchical level (fig. 5 and supplementary table S5, Supplementary Material online). fineSTRUCTURE was a two-stage analysis consisting of in silico chromosome painting that created a coancestry matrix containing the number of blocks copied from each donor strain to each recipient strain. The fineSTRUCTURE algorithm enabled use of this coancestry matrix to conduct model-based linkage clustering and assign individual strains to 18 populations (fig. 6).

As expected, both methods mapped populations to distinct clades of the phylogenetic tree (figs. 1 and 2). fineSTRUCTURE grouped *C. pneumoniae* strains into two populations: The

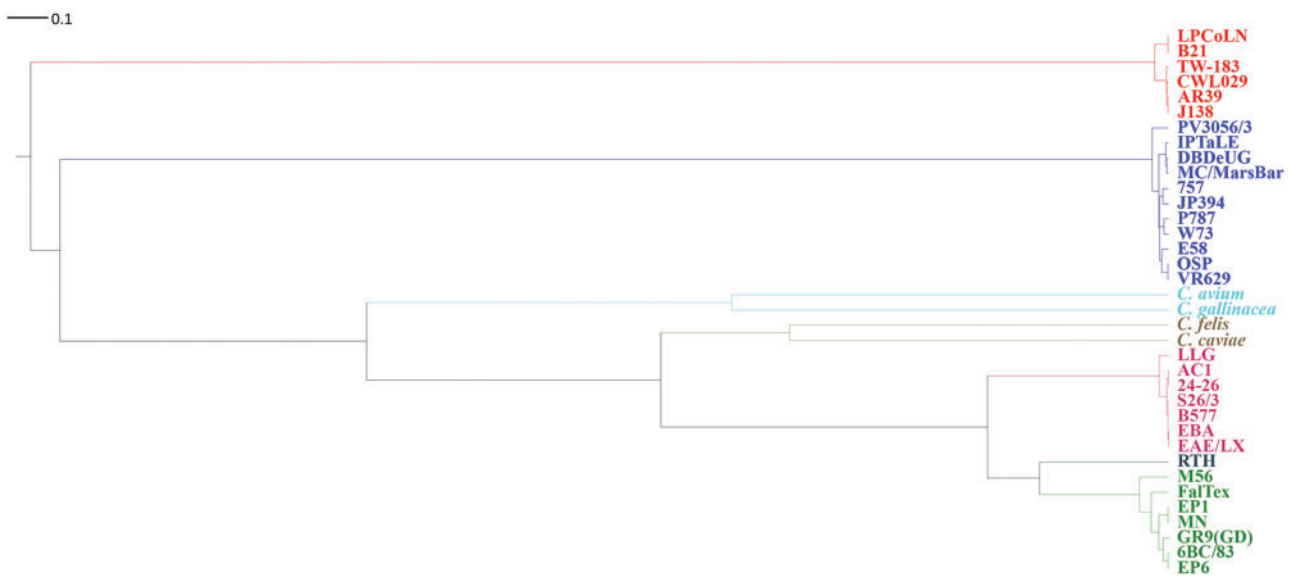


Fig. 2.—Clonal genealogy inferred by ClonalFrame. Whole-genome alignment data for the 36 *Chlamydiaceae* genomes in supplementary table S2, Supplementary Material online, except for the sole representative of the *C. felis*, *C. caviae*, *C. avium*, and *C. gallinacea* species, were used for analysis. The branch lengths are shown in coalescent units of time.

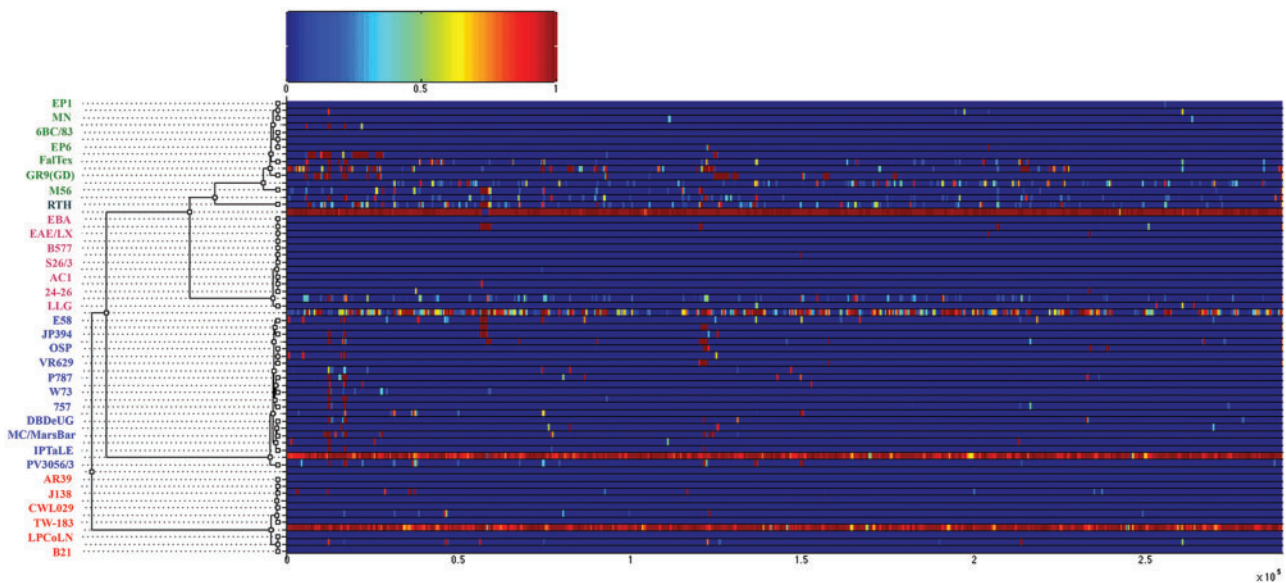


Fig. 3.—Results of the ClonalFrame analysis on an alignment of 32 chlamydial genomes. Inferred clonal genealogy is shown on the left. Each branch of the tree corresponds to a row of the heat map, which is horizontally aligned according to the core MAUVE whole-genome alignment. Each row shows the posterior probability of recombination estimated by ClonalFrame on the corresponding branch (y axis) and along positions of the alignment (x axis; $\times 10^5$ bp). *Chlamydia psittaci*, *C. abortus*, *C. pecorum* and *C. pneumoniae* strains are in green, magenta, blue and red, respectively. The *C. psittaci* RTH is shown in dark blue.

LPCoLN and B21 strains isolated from lower mammals; and the remaining four strains isolated from humans. However, BAPS clustered *C. pneumoniae* strains into three groups: TW-183 and CWL029 in one group; J138 and AR39 into a second group; and B21 and LPCoLN into a third group (fig. 5). The 11

C. pecorum strains were segregated into 5, 5, and 6 groups by phylogeny, fineSTRUCTURE, and BAPS analysis, respectively. The three koala-infecting *C. pecorum* strains, MC/MarsBar, DBDeUG and IPTaLE, were grouped together in one well-defined clade by all methods. *Chlamydia pecorum* sheep

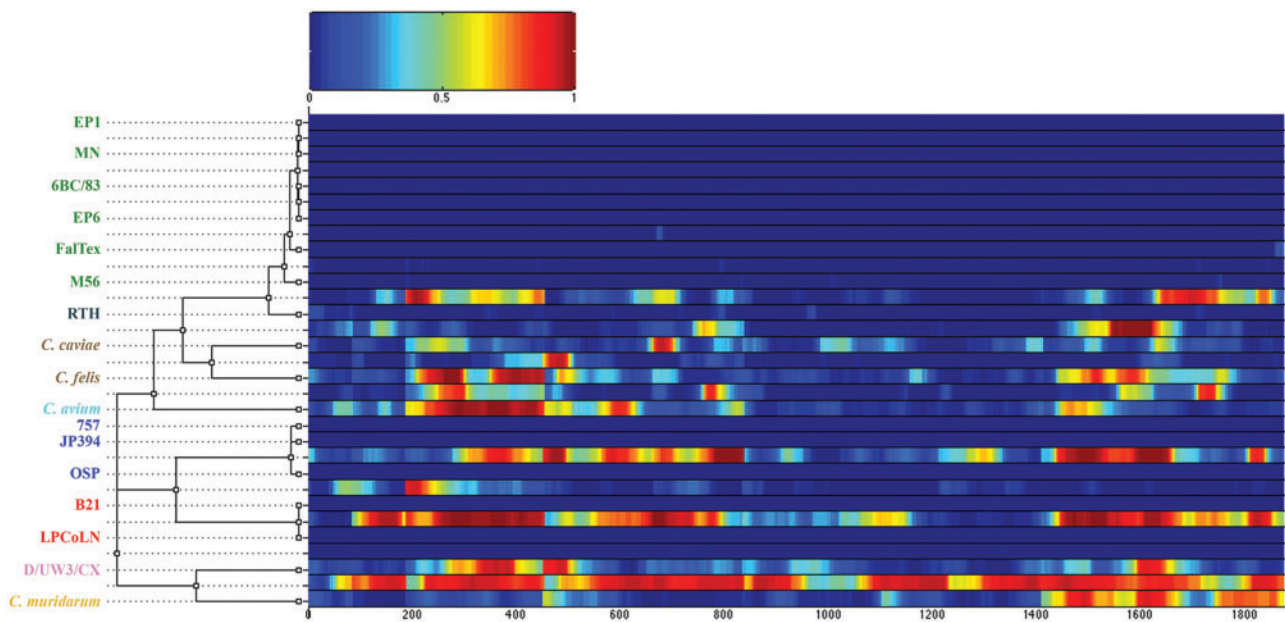


FIG. 4.—Results of the ClonalFrame analysis on an alignment of 17 *Chlamydiaceae* plasmids. The inferred clonal genealogy is shown on the left. Each branch of the tree corresponds to a row of the heat map, which is horizontally aligned according to the core MAUVE whole-genome alignment. Each row of the heat map shows the posterior probability of recombination estimated by ClonalFrame on the corresponding branch (*y* axis) and along the positions of the alignment (*x* axis). *C. psittaci*, *C. pecorum*, and *C. pneumoniae* species are in green, magenta, blue and red, respectively.

strains W73, P787, 757, and JP396 that clustered together by fineSTRUCTURE were regrouped into two distinct clusters at the fourth finest hierarchical level by BAPS. The patterns of admixture for those strains inferred by BAPS support this partition (see below; fig. 5 and [supplementary table S5, Supplementary Material](#) online). The *C. pecorum* bovine strains E58 and PV3056/3 were grouped into distinct single strain clusters by both fineSTRUCTURE and BAPS. E58 did not group with the clade formed by sheep strains VR629 and OSP in the ML phylogeny but grouped with JP396 by ClonalFrame phylogenetic analysis. Even though the phylogenetic methods and fineSTRUCTURE appeared to have three distinct clades for *C. abortus* with the LLG strain as an outgroup, BAPS inferred the presence of four clusters, placing the B577 strain as a distinct cluster (figs. 5 and 6; [supplementary table S5, Supplementary Material](#) online).

The new *C. psittaci* EP1 strain grouped with the MN strain forming a distinct clade by all three methods. In a previous analysis (Read et al. 2013), the MN strain did not form a distinct clade. The other novel *C. psittaci* strain, EP6, isolated from a human grouped with the 6BC/83 strain by phylogeny, fineSTRUCTURE and BAPS hierarchical analysis ([supplementary table S5, Supplementary Material](#) online), indicating that this strain is also part of the recent clonal expansion of *C. psittaci* in the United States. Interestingly, the *C. psittaci* RTH strain was assigned into a single cluster by both population structure methods.

The newly identified species *C. avium* and *C. gallinacea* grouped together forming a single clade by ML-based phylogenetic inference and by fineSTRUCTURE, indicating that they may be part of a similar population despite the ANI score ([supplementary table S3, Supplementary Material](#) online) that puts them into different species groups (fig. 6). Similarly, *C. caviae* and *C. felis* formed a single clade (fig. 6).

Signatures of Admixture Suggest a Genetic and/or Ecological Barriers between Species Groups

Based on the fineSTRUCTURE coancestry matrix visualized as a heatmap (fig. 6), there appeared to be many events of genetic exchange happening among the different subgroups within and across each of the species. The color of each cell of the matrix indicated the expected number of genetic markers imported from the donor (*x* axis) to a recipient genome (*y* axis). *Chlamydia psittaci* RTH strain received DNA sequences from all *C. abortus* and *C. psittaci* strains, primarily *C. abortus* LLG, *C. psittaci* FalTex, and *C. psittaci* M56 genomes. Admixtures of DNA exchange were previously observed by STRUCTURE analysis (Read et al. 2013) between the RTH strain and *C. psittaci* strains M56, FalTex, and MN. The subgroup of *C. felis* and *C. caviae* received DNA from all *C. abortus* and *C. psittaci* strains along with smaller amounts from *C. pneumoniae* and *C. pecorum* strains. This trend was also observed in the *C. avium* and *C. gallinacea* subgroup, but to a lower

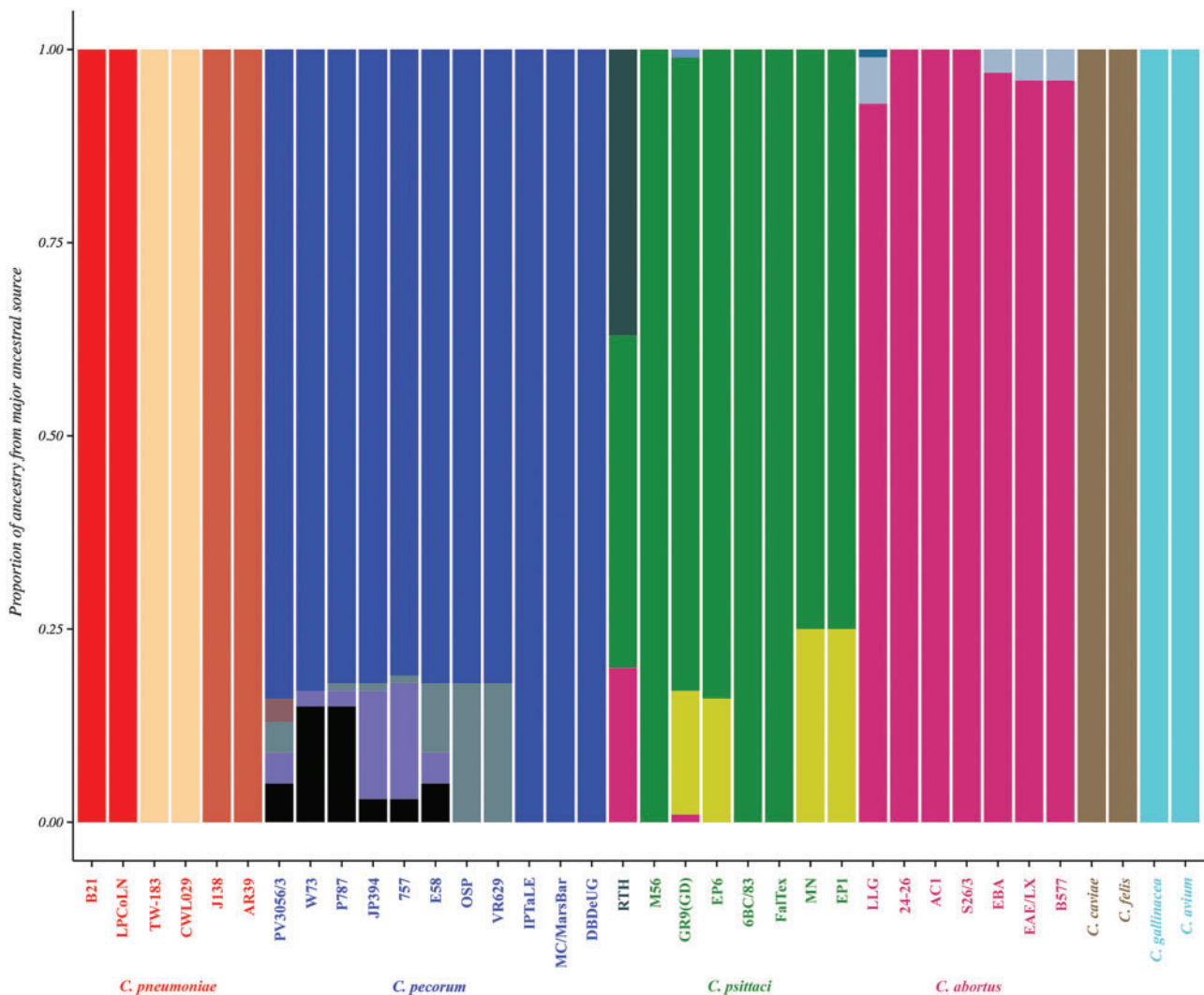


Fig. 5.—Admixture analysis of *Chlamydiaceae*. Mixture and admixture analyses were conducted using BAPS. Each color represents one of the genetically differentiated groups, and each vertical colored bar corresponds to one isolate. When a vertical bar shows two or more colors, each color corresponds to one of the groups, showing evidence for admixture; the proportion of every color in the bar reflects the proportion of the isolate coming from the group represented by that particular color. For example, the ancestral source of strain GR9(GD) consists predominately of *C. psittaci* (green) but also contains sources from *C. abortus* (magenta) and from two different external sources (yellow and pale blue).

extent; the RTH strain was the most significant donor to that subgroup (fig. 6).

The fineSTRUCTURE algorithm assumed that all genetic exchanges occurred within the sequenced strains, whereas ClonalFrame allowed recombination events from “outside” the sample of genomes under consideration (although they do not model the source). Figure 5 shows the proportion of ancestry from all major ancestral sources in separate colors for each species. There were no signs of admixture signals in the three distinct ancestral populations of *C. pneumoniae*. Except for the koala-infecting *C. pecorum* strains, which had a 100% proportion of ancestry from a single ancestral source of *C. pecorum* (shown in blue), admixture signals from four

other unknown/external ancestral populations were estimated (colors other than blue). For *C. psittaci* strains, evident admixture signals were estimated from at least two other unknown ancestral populations (gray and yellow) along with exchanges from *C. abortus* ancestral population (magenta). The *C. psittaci* RTH strain had evidence of admixture signals from three ancestral populations; 43% from *C. psittaci* (green), 20% from *C. abortus* (magenta) sources, and 37% from an unknown ancestral population (gray). *Chlamydia abortus* strains also showed events of admixtures from two other unknown ancestral populations, but at very low proportions. In the BAPS model, the two novel species, *C. gallinacea* and *C. avium* did not show any evidence of admixtures; both were estimated to

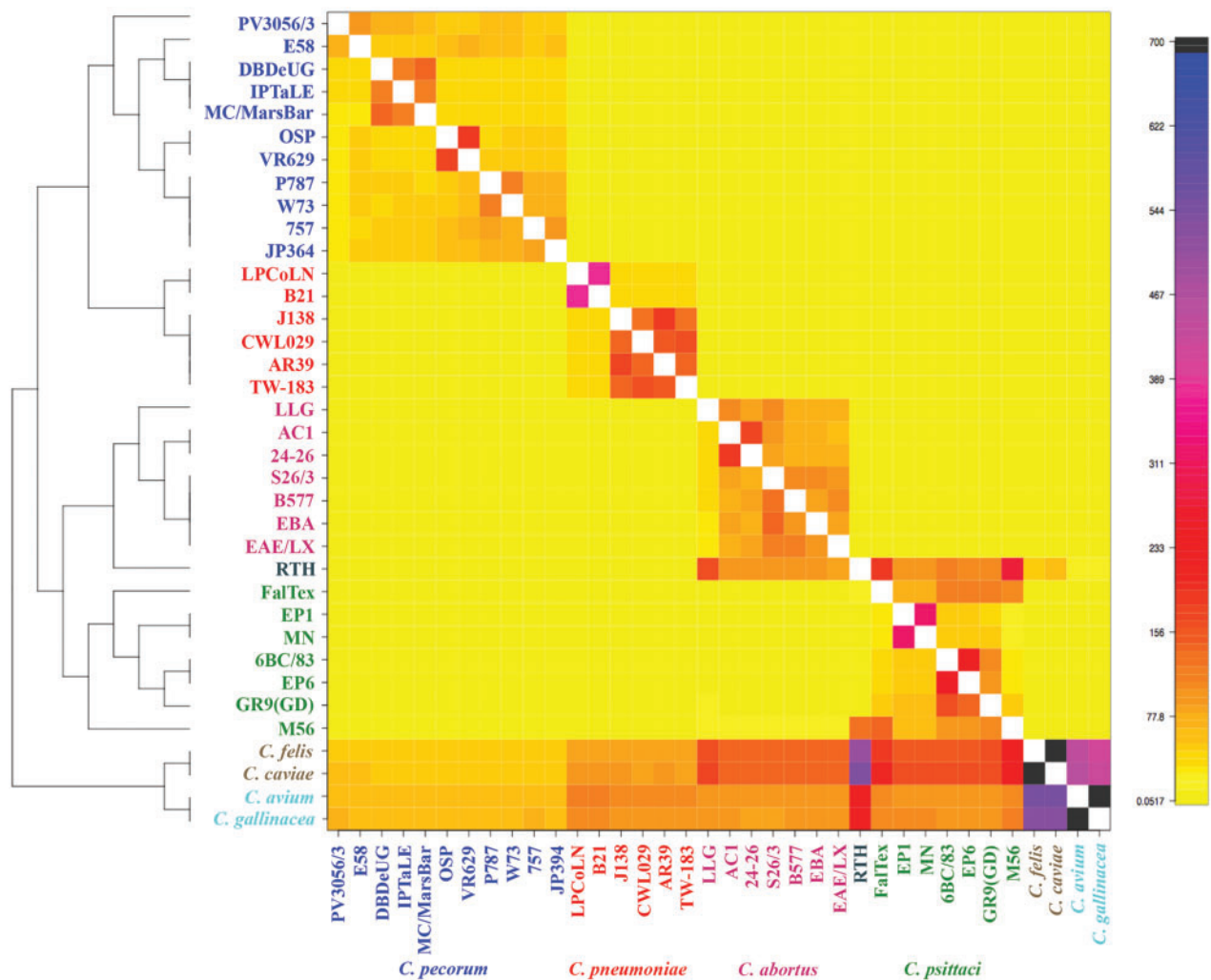


FIG. 6.—ChromPainter co-ancestry matrix with population structure assignment based on fineSTRUCTURE analysis. The color of each cell of the matrix indicates the expected number of DNA chunks copied from a donor genome (x axis) to a recipient genome (y axis).

have 100% ancestry from a single ancestral source. Similarly, *C. caviae* and *C. felis* did not have evidence of exchange from other *Chlamydiaceae* species (fig. 5).

We assigned the origin of each recombination event identified by ClonalFrame by postprocessing the output (see Materials and Methods) (supplementary table S6, Supplementary Material online). Figure 7 summarizes the flux of recombination among the three major *Chlamydiaceae* species, *C. abortus*, *C. psittaci* and *C. pecorum*, that showed significant amounts of recombination. We also estimated the number and origin of recombination events for the *C. psittaci* RTH strain (fig. 7).

Recombination events occurred on the ancestral nodes and are still present in each of the species. For *C. pecorum*, the origins for all 18 recombination events were attributed to an unknown “external” source (probably as yet undiscovered

Chlamydiaceae species), which corroborated with the results from BAPS. There were 21 recombination events where the origin was from an unknown external source that occurred at the common ancestral nodes of *C. abortus* and *C. psittaci*. All 21 recombination events were also detected in the *C. psittaci* RTH genome. There were 14 recombination events in *C. psittaci* whose origin of recombination was assigned to *C. abortus*. These data suggested that DNA exchanges were occurring between *C. abortus*, *C. psittaci*, and the RTH strains. For the plasmids, there were 18 recombination events identified. Four recombination events that occurred on the ancestral node of *C. trachomatis* and *C. muridarum* were traced back to import from the *C. pneumoniae* plasmid, suggesting that the event of acquisition of plasmids in *C. trachomatis* and *C. muridarum* might not be an ancient evolutionary event but a recent one, and most probably acquired from *C. pneumoniae*.

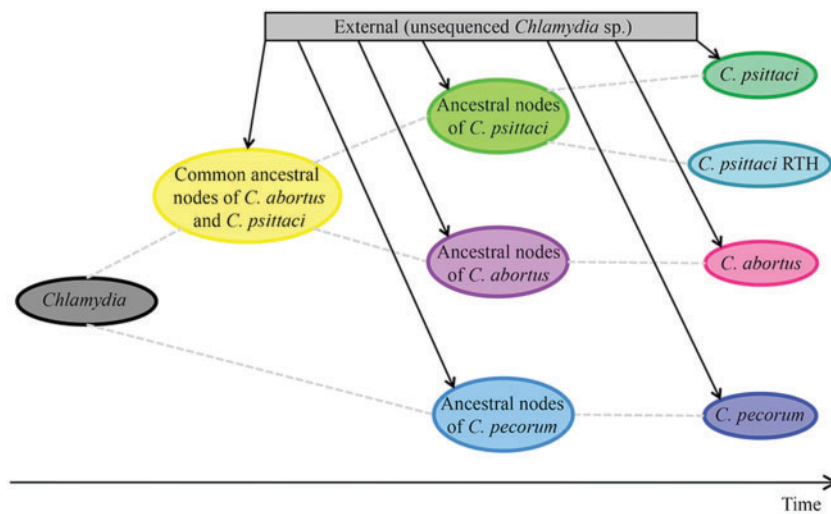


Fig. 7.—Recombination flux between the *Chlamydiaceae* species. Based on ClonalFrame analysis, the flux of recombination among the three major *Chlamydiaceae* species, *C. abortus*, *C. psittaci* and *C. pecorum*, is shown on an axis of time. Dashed lines indicate vertical inheritance of the *Chlamydiaceae* genomes. Solid lines indicate heritage from an external source.

We were not able to assign the origins of imports for the rest of the plasmid recombination events.

Discussion

One of the main aims of this work was to present a detailed analysis of the population genomics of *Chlamydiaceae* species isolated from avian, mammalian and human hosts and, in particular, for those in the *Chlamydophila* lineage. Taking into consideration that species designation for this lineage is often based on clinical symptoms (e.g., *C. abortus* is found in abortion) rather than molecular analysis, the fact that five of the ten novel genomes sequenced in this work were originally typed as *C. abortus* but were in fact either *C. psittaci* or *C. pecorum* indicates that we will remain ignorant of the true diversity of *Chlamydiaceae* without the use of appropriate methodology. The discovery of genetically admixed strains illustrates the importance of whole-genome as opposed to partial gene analysis. Indeed, it is likely that many of the strains not yet sequenced are mistyped.

Here, we extend our knowledge of *C. abortus* by showing it to be a highly monomorphic group with very similar gene content. *Chlamydia abortus* is the most recently emerged species and closely related to the more diverse *C. psittaci*, not only in terms of genetics but also host association and disease pathology. The high dN/dS ratios for *C. abortus* have previously been reported in other rapidly evolving pathogens such as *Clostridium difficile* (He et al. 2010) and could be caused by ongoing adaptation of the *C. abortus* population to a new pathogenic lifestyle (Shapiro et al. 2009). Furthermore, the low-level of recombination relative to other species and evidence for adaptation to the placenta in *C. abortus* echo

evolutionary processes seen in other recently emerged, highly virulent pathogens with restricted ecological niches, such as *Bacillus anthracis* and *Mycobacterium tuberculosis* (Liu et al. 2006; Didelot and Maiden 2010; Zwick et al. 2012).

We showed that gene-flow occurred within the species and between *C. psittaci* and *C. abortus*. However, there was no evidence here of gene-flow between or among *C. felis*, *C. caviae*, *C. avium*, *C. gallinacea*, *C. pecorum*, and *C. pneumoniae*. Homologous recombination among *Chlamydiaceae* species has emerged as a central feature from comparative genomics studies in the past few years (Gomes et al. 2007; Jeffrey et al. 2010; Harris et al. 2012; Joseph et al. 2012; Read et al. 2013). The exact mechanism for DNA transfer between *Chlamydiaceae* infected cells is unknown, but laboratory studies have shown that recombination can occur after host cell coinfection (DeMars and Weinfurter 2008; Suchland et al. 2009; Srinivasan et al. 2012). There is no absolute requirement for transducing bacteriophage. However, that does not rule out a role in some exchanges since a number of bacteriophages have been identified for *C. psittaci*, *C. abortus*, *C. felis*, *C. caviae*, *C. pecorum*, and *C. pneumoniae* (Pawlikowska-Warych et al. 2015).

Not all species are permissive to interspecies recombination. Barriers to recombination have been documented for *C. caviae* and *C. suis*; *C. caviae* and *C. trachomatis* (Suchland et al. 2009); and *C. muridarum* and *C. psittaci* (Millman et al. 2001). Without additional laboratory experiments, we do not know whether the lack of recombination discovered in the present study between *C. psittaci* (except for the RTH strain) and *C. abortus*, and *C. pecorum* and *C. pneumoniae* is primarily the result of an ecological or a genetic barrier. In the latter case, the reduced efficiency of mismatch repair

following homologous recombination between divergent nucleotide sequences may be a major factor (Matic et al. 1995).

With the proviso that the *r/m* statistic for recombination frequency has several well-known ascertainment biases (Shapiro 2014), it may still be significant that the highest rates of exchange are found in *C. psittaci* strains. *Chlamydia psittaci* has an extremely broad avian host range encompassing 460 species in 30 orders including 9 domestic species (e.g., turkey, duck, pigeon, quail, chicken, goose, peafowl) and is most commonly detected in the orders Psittaciformes, followed by Lariformes (gulls), Alciformes (alks), Sphenisciformes (penguins), and Anseriformes (ducks and geese) (Kaleta and Taday 2003). It is also noteworthy that the most highly genetically admixed *C. psittaci* strain, RTH, was discovered in a wild bird-of-prey. Similar to the RTH strain, other *Chlamydiaceae* species have been isolated from a number of other predatory birds (Kaleta and Taday 2003; Van Loock et al. 2003; Schettler et al. 2003; Blomqvist et al. 2012), some of which do not fit easily within either the *C. psittaci* or *C. abortus* species, based on limited genetic data. 16S rRNA and *ompA* gene analysis of four samples revealed one novel *Chlamydia* species as well as two novel *C. psittaci* strains in peregrine falcons and white-tailed sea eagles in Sweden (Blomqvist et al. 2012). Other strains that are seemingly intermediate between *C. psittaci* and *C. abortus* include the R54 strain from the subantarctic brown skua (Herrmann et al. 2000), the 84/2334 strain isolated from a yellow-crowned amazon in Germany (Vanrompay et al. 1997), the parakeet strains Prk/Daruma (Fukushi and Hirai 1988) and strain VS225 (Van Loock et al. 2003) isolated in Japan, and in 1991 in Laredo, TX, respectively. It would be informative to see whether isolates from these and other birds-of-prey harbor diverse strains with evidence of recent recombination between species.

Theoretically, infection by different *Chlamydiaceae* species either simultaneously or sequentially would facilitate transformation and homologous recombination (Gomes et al. 2007). The red-tailed hawk (*Buteo jamaicensis*), from which the RTH strain was isolated, is one of the most common and widely distributed raptors in North America, living in various habitats ranging from forestland to agricultural and even urban landscapes (Fitch et al. 1946; Stout et al. 2006). This variety of nesting and hunting grounds also requires great flexibility in their diet. Red-tailed hawks prey on small mammals (squirrels, mice, and rabbits), birds, such as pigeons and pheasants (Gates 1972), as well as reptiles including snakes and lizards (Fitch et al. 1946). They also feed on carrion (Marti and Kochert 1995) and, if living near agricultural landscapes, may seize dead chickens that were spread on the fields with manure (Oriens and Kuhlman 1956). Because a variety of *Chlamydiaceae* species infect the intestinal tract of many avian and lower mammalian species, infected feces are ubiquitous in these environments and available for ingestion along with carrion or other animals. These dietary habits are

important because horizontal gene transfer of entire metabolic pathways, fitness and virulence factors, as well as antibiotic resistance genes, has been reported in the gut ecosystem, particularly among closely related bacterial species (Stecher et al. 2013). These findings lend support to our hypothesis that the RTH and other raptors serve as a nexus for *Chlamydiaceae* evolution.

Based on the plasmid phylogeny, each species separated into monophyletic clades (supplementary fig. S1, Supplementary Material online). Surprisingly, the plasmids of *C. pneumoniae* and *C. pecorum* were ancestral to *C. trachomatis* and *C. muridarum*, whereas these four species shared a more recent ancestor than the other *Chlamydia* species. The unexpected similarity of plasmid genes in the *C. trachomatis* and *C. muridarum* lineage to *C. pneumoniae* and *C. pecorum* suggest a potential ancestral transfer event. *Chlamydia trachomatis* and *C. pneumoniae* are both human pathogens that infect the respiratory tract (Webley et al. 2009), providing an opportunity for genetic exchange. Mice could potentially acquire *C. pecorum* with its plasmid from feces that are ubiquitous in a farmyard setting. This would create the potential opportunity for DNA/plasmid exchange with *C. muridarum* in the intestinal tract.

This work also raises issues in terms of how we define *Chlamydia* species given the *Chlamydiaceae* population structure. *Chlamydia avium* and *C. gallinacea* have recently been adopted as new species based on ANI scores of at most 94% with the closest other genome (Sachse et al. 2014). The cutoff for strain assignment to the same species has been suggested to be approximately 95–96% ANI (Richter and Rosselló-Móra 2009). However, population structure results place them in the same genetic group as another existing species (figs. 5 and 6). *Chlamydia avium* and *C. gallinacea* (and *C. caviae/C. felis*) could be divergent clades of genetic congruent species (fig. 2) or have undergone sympatric speciation with ongoing frequent genetic exchanges.

Finally, the *C. psittaci* RTH strain has an ANI relationship that suggests that it is a separate species from both *C. psittaci* and *C. abortus* and may be a representative of a new *Chlamydia* species altogether. Although there is evidence of DNA exchange, the RTH strain still has unique population markers and possibly a unique ecology that would qualify it to be treated as a distinct species. The molecular clock data would support this contention as the RTH strain is ancestral to *C. abortus*. As with many other stories concerning *Chlamydiaceae*, the answers are likely to come from further genomic sequencing and bioinformatics efforts.

Conclusion

The family *Chlamydiaceae* are bacteria that cause mild to severe diseases in humans, livestock and wild animals, such as koalas, frogs, and birds-of-prey. The estimated economic burden of these diseases is over \$10 billion annually. Given

their zoonotic potential for ocular, respiratory, and systemic diseases as well as abortion, we examined the evolutionary dynamics of *Chlamydia* species using genomes from 36 isolates of diverse origins. Analyses revealed that *C. abortus* is the most recently emerged species with significantly lower recombination compared with all other species and lacks the virulence-associated plasmid. Through gene-flow analysis, the RTH isolate was found to have extensive genomic admixture from chlamydial and external sources, and may be a member of a novel intermediary species linking *C. psittaci* to *C. abortus* evolution. Predators such as birds-of-prey may serve as incubator hosts that acquire chlamydiae and other pathogens in their guts resulting in species emergence by recombination.

Supplementary Material

Supplementary figures S1–S4 and tables S1–S6 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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Literature Cited

- Azuma Y, et al. 2006. Genome sequence of the cat pathogen, *Chlamydomydia felis*. *DNA Res.* 13:15–23.
- Bachmann NL, et al. 2015. Culture-independent genome sequencing of clinical samples reveals an unexpected heterogeneity of infections by *Chlamydia pecorum*. *J Clin Microbiol.* 53:1573–1581.
- Bachmann NL, Fraser TA, et al. 2014. Comparative genomics of koala, cattle and sheep strains of *Chlamydia pecorum*. *BMC Genomics* 15:667.
- Bachmann NL, Polkinghorne A, Timms P. 2014. *Chlamydia* genomics: providing novel insights into chlamydial biology. *Trends Microbiol.* 22:464–472.
- Blomqvist M, et al. 2012. *Chlamydia psittaci* in birds of prey, Sweden. *Infect Ecol Epidemiol.* 2.
- Bodetti TJ, et al. 2002. Molecular evidence to support the expansion of the host range of *Chlamydomydia pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst Appl Microbiol.* 25:146–152.
- Castillo-Ramírez S, et al. 2012. Phylogeographic variation in recombination rates within a global clone of methicillin-resistant *Staphylococcus aureus*. *Genome Biol.* 13:R126.
- Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol.* 17:540–552.
- Cheng L, Connor TR, Sirén J, Aanensen DM, Corander J. 2013. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. *Mol Biol Evol.* 30:1224–1228.
- Collingro A, et al. 2011. Unity in variety—the pan-genome of the *Chlamydiaceae*. *Mol Biol Evol.* 28:3253–3270.
- Corander J, Marttinen P. 2006. Bayesian identification of admixture events using multilocus molecular markers. *Mol Ecol.* 15:2833–2843.
- Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14:1394–1403.
- Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147.
- Dean D, Rothschild J, Ruettinger A, Kandel RP, Sachse K. 2013. Zoonotic *Chlamydiaceae* species associated with trachoma, Nepal. *Emerg Infect Dis.* 19:1948–1955.
- DeMars R, Weinfurter J. 2008. Interstrain gene transfer in *Chlamydia trachomatis* in vitro: mechanism and significance. *J Bacteriol.* 190:1605–1614.
- Didelot X, et al. 2011. Recombination and population structure in *Salmonella enterica*. *PLoS Genet.* 7:e1002191.
- Didelot X, Falush D. 2007. Inference of bacterial microevolution using multilocus sequence data. *Genetics* 175:1251–1266.
- Didelot X, Lawson D, Darling A, Falush D. 2010. Inference of homologous recombination in bacteria using whole-genome sequences. *Genetics* 186:1435–1449.
- Didelot X, Maiden MCJ. 2010. Impact of recombination on bacterial evolution. *Trends Microbiol.* 18:315–322.
- Donati M, et al. 2014. Genome sequence of *Chlamydia suis* MD56, isolated from the conjunctiva of a weaned piglet. *Genome Announc.* 2:e00425–14.
- Drummond AJ, Nicholls GK, Rodrigo AG, Solomon W. 2002. Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics* 161:1307–1320.
- Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–1797.
- Everett KD, Bush RM, Andersen AA. 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol.* 49(Pt 2):415–440.
- Fitch HS, Swenson F, Tillotson DF. 1946. Behavior and food habits of the red-tailed hawk. *Condor* 48:205–237.
- Fukushi H, Hirai K. 1988. Immunochemical diversity of the major outer membrane protein of avian and mammalian *Chlamydia psittaci*. *J Clin Microbiol.* 26:675–680.
- Fukushi H, Hirai K. 1992. Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. *Int J Syst Bacteriol.* 42:306–308.
- Gates JM. 1972. Red-tailed hawk populations and ecology in east-central Wisconsin. *Wilson Bull.* 84:421–433.
- Gomes JP, et al. 2007. Evolution of *Chlamydia trachomatis* diversity occurs by widespread interstrain recombination involving hotspots. *Genome Res.* 17:50–60.
- Greub G. 2010. International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of the *Chlamydiae* Minutes of the inaugural closed meeting, 21 March 2009, Little Rock, AR, USA. *Int J Syst Evol Microbiol.* 60:2691–2693.
- Harkinezhad T, et al. 2009. Prevalence of *Chlamydomydia psittaci* infections in a human population in contact with domestic and companion birds. *J Med Microbiol.* 58:1207–1212.

- Harris SR, et al. 2012. Whole-genome analysis of diverse *Chlamydia trachomatis* strains identifies phylogenetic relationships masked by current clinical typing. *Nat Genet.* 44:413–419, S1.
- He M, et al. 2010. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A.* 107:7527–7532.
- Herrmann B, Rahman R, Bergström S, Bonnedahl J, Olsen B. 2000. *Chlamydophila abortus* in a Brown skua (*Catharacta antarctica lonnbergi*) from a subantarctic island. *Appl Environ Microbiol.* 66:3654–3656.
- Hershberg R, Petrov DA. 2010. Evidence that mutation is universally biased towards AT in bacteria. *PLoS Genet.* 6:e1001115.
- Jeffrey BM, et al. 2010. Genome sequencing of recent clinical *Chlamydia trachomatis* strains identifies loci associated with tissue tropism and regions of apparent recombination. *Infect Immun.* 78:2544–2553.
- Joseph SJ, Didelot X, Gandhi K, Dean D, Read TD. 2011. Interplay of recombination and selection in the genomes of *Chlamydia trachomatis*. *Biol Direct.* 6:28.
- Joseph SJ, et al. 2012. Population genomics of *Chlamydia trachomatis*: insights on drift, selection, recombination, and population structure. *Mol Biol Evol.* 29:3933–3946.
- Joseph SJ, Read TD. 2012. Genome-wide recombination in *Chlamydia trachomatis*. *Nat Genet.* 44:364–366.
- Kaleta EF, Taday EMA. 2003. Avian host range of *Chlamydophila* spp. based on isolation, antigen detection and serology. *Avian Pathol.* 32:435–461.
- Li L, Stoeckert CJ Jr, Roos DS. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* 13:2178–2189.
- Liu X, Gutacker MM, Musser JM, Fu Y-X. 2006. Evidence for recombination in *Mycobacterium tuberculosis*. *J Bacteriol.* 188:8169–8177.
- Longbottom D, Coulter LJ. 2003. Animal chlamydioses and zoonotic implications. *J Comp Pathol.* 128:217–244.
- Marti CD, Kochert MN. 1995. Are red-tailed hawks and great horned owls diurnal-nocturnal dietary counterparts? *Wilson Bull.* 107:615–628.
- Matic I, Rayssiguier C, Radman M. 1995. Interspecies gene exchange in bacteria: the role of SOS and mismatch repair systems in evolution of species. *Cell* 80:507–515.
- Millman KL, Tavare S, Dean D. 2001. Recombination in the *ompA* Gene but not the *omcB* gene of *Chlamydia* contributes to serovar-specific differences in tissue tropism, immune surveillance, and persistence of the organism. *J Bacteriol.* 183:5997–6008.
- Mohamad KY, Rodolakis A. 2010. Recent advances in the understanding of *Chlamydophila pecorum* infections, sixteen years after it was named as the fourth species of the Chlamydiaceae family. *Vet Res.* 41:27.
- Myers GSA, et al. 2009. Evidence that human *Chlamydia pneumoniae* was zoonotically acquired. *J Bacteriol.* 191:7225–7233.
- Orians G, Kuhlman F. 1956. Red-tailed hawk and horned owl populations in Wisconsin. *Condor* 58:371–385.
- Pawlikowska-Warych M, Śliwa-Dominiak J, Deptuła W. 2015. Chlamydial plasmids and bacteriophages. *Acta Biochim Pol.* 62:1–6.
- Read TD, et al. 2000. Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res.* 28:1397–1406.
- Read TD, et al. 2003. Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the Chlamydiaceae. *Nucleic Acids Res.* 31:2134–2147.
- Read TD, et al. 2013. Comparative analysis of *Chlamydia psittaci* genomes reveals the recent emergence of a pathogenic lineage with a broad host range. *MBio* 4:e00604–12.
- Reinhold P, Sachse K, Kaltenboeck B. 2011. *Chlamydiaceae* in cattle: commensals, trigger organisms, or pathogens? *Vet J.* 189:257–267.
- Richter M, Rosselló-Móra R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A.* 106:19126–19131.
- Rocha EPC, et al. 2006. Comparisons of dN/dS are time dependent for closely related bacterial genomes. *J Theor Biol.* 239:226–235.
- Rockey DD, Heinzen RA, Hackstadt T. 1995. Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol Microbiol.* 15:617–626.
- Roulis E, Polkinghorne A, Timms P. 2013. *Chlamydia pneumoniae*: modern insights into an ancient pathogen. *Trends Microbiol.* 21:120–128.
- Sachse K, et al. 2014. Evidence for the existence of two new members of the family *Chlamydiaceae* and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. *Syst Appl Microbiol.* 37:79–88.
- Sachse K, et al. 2015. Emendation of the family *Chlamydiaceae*: proposal of a single genus, *Chlamydia*, to include all currently recognized species. *Syst Appl Microbiol.* 38:99–103.
- Sait M, et al. 2014. Genome sequencing and comparative analysis of three *Chlamydia pecorum* strains associated with different pathogenic outcomes. *BMC Genomics* 15:23.
- Schautteet K, Vanrompay D. 2011. *Chlamydiaceae* infections in pig. *Vet Res.* 42:29.
- Schettler E, et al. 2003. Newcastle disease virus and *Chlamydia psittaci* in free-living raptors from eastern Germany. *J Wildl Dis.* 39:57–63.
- Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30:2068–2069.
- Shao R, Hu J, Billig H. 2013. Toward understanding *Chlamydia* infection-induced infertility caused by dysfunctional oviducts. *J Infect Dis.* 208:707–709.
- Shapiro BJ. 2014. Signatures of natural selection and ecological differentiation in microbial genomes. *Adv Exp Med Biol.* 781:339–359.
- Shapiro BJ, David LA, Friedman J, Alm EJ. 2009. Looking for Darwin's footprints in the microbial world. *Trends Microbiol.* 17:196–204.
- Snipen L, Liland KH. 2015. micromanmicropan: an R-package for microbial pan-genomics. *BMC Bioinformatics* 16:79.
- Somboonna N, et al. 2011. Hypervirulent *Chlamydia trachomatis* clinical strain is a recombinant between lymphogranuloma venereum (L2) and D lineages. *MBio* 2:e00045–11.
- Srinivasan T, et al. 2012. In vitro recombinants of antibiotic-resistant *Chlamydia trachomatis* strains have statistically more breakpoints than clinical recombinants for the same sequenced loci and exhibit selection at unexpected loci. *J Bacteriol.* 194:617–626.
- Stamatakis A, et al. 2012. RAXML-Light: a tool for computing terabyte phylogenies. *Bioinformatics* 28:2064–2066.
- Stecher B, Maier L, Hardt W-D. 2013. “Blooming” in the gut: how dysbiosis might contribute to pathogen evolution. *Nat Rev Microbiol.* 11:277–284.
- Stout WE, Temple SA, Cary JR. 2006. Landscape features of red-tailed hawk nesting habitat in an urban/suburban environment. *J Raptor Res.* 40:181–192.
- Suchland RJ, Sandoz KM, Jeffrey BM, Stamm WE, Rockey DD. 2009. Horizontal transfer of tetracycline resistance among *Chlamydia* spp. in vitro. *Antimicrob Agents Chemother.* 53:4604–4611.
- Suyama M, Torrents D, Bork P. 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* 34:W609–W612.
- Tang J, Hanage WP, Fraser C, Corander J. 2009. Identifying currents in the gene pool for bacterial populations using an integrative approach. *PLoS Comput Biol.* 5:e1000455.
- Thomson NR, et al. 2005. The *Chlamydophila abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. *Genome Res.* 15:629–640.
- Van Look M, et al. 2003. Missing links in the divergence of *Chlamydophila abortus* from *Chlamydophila psittaci*. *Int J Syst Evol Microbiol.* 53:761–770.
- Vanrompay D, Butaye P, Sayada C, Ducatelle R, Haesebrouck F. 1997. Characterization of avian *Chlamydia psittaci* strains using *omp1*

- restriction mapping and serovar-specific monoclonal antibodies. *Res Microbiol.* 148:327–333.
- Voigt A, Schöfl G, Saluz HP. 2012. The *Chlamydia psittaci* genome: a comparative analysis of intracellular pathogens. *PLoS One* 7:e35097.
- Webley WC, et al. 2009. Occurrence of *Chlamydia trachomatis* and *Chlamydia pneumoniae* in paediatric respiratory infections. *Eur Respir J.* 33:360–367.
- Yahara K, et al. 2013. Chromosome painting in silico in a bacterial species reveals fine population structure. *Mol Biol Evol.* 30:1454–1464.
- Zwick ME, et al. 2012. Genomic characterization of the *Bacillus cereus* sensu lato species: backdrop to the evolution of *Bacillus anthracis*. *Genome Res.* 22:1512–1524.

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