Genomic analysis of the causative agents of coccidiosis in domestic chickens

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Global production of chickens has trebled in the past two decades and they are now the most important source of dietary animal protein worldwide. Chickens are subject to many infectious diseases that reduce their performance and productivity. Coccidiosis, caused by apicomplexan protozoa of the genus *Eimeria*, is one of the most important poultry diseases. Understanding the biology of *Eimeria* parasites underpins development of new drugs and vaccines needed to improve global food security. We have produced annotated genome sequences of all seven species of *Eimeria* that infect domestic chickens, which reveal the full extent of previously described repeat-rich and repeat-poor regions and show that these parasites possess the most repeat-rich proteomes ever described. Furthermore, while no other apicomplexan has been found to possess retrotransposons, *Eimeria* is home to a family of chromoviruses. Analysis of *Eimeria* genes involved in basic biology and host-parasite interaction highlights adaptations to a relatively simple developmental life cycle and a complex array of co-expressed surface proteins involved in host cell binding.

[Supplemental material is available for this article.]

Chickens are the world's most popular food animal, and the development of improved drugs and vaccines to combat poultry diseases are vital for worldwide food security. Protozoan parasites of the genus *Eimeria* cause coccidiosis, a ubiquitous intestinal disease of livestock that has major impacts on animal welfare and agro-economics. It is a particularly acute problem in poultry where infections can cause high mortality and are linked to poor performance and productivity. *Eimeria* belong to the phylum Apicomplexa, which includes thousands of parasitic protozoa such as *Plasmodium* species that cause malaria, and the widely disseminated zoonotic pathogen *Toxoplasma gondii*. *Eimeria* species have a direct oral-fecal life cycle that facilitates their rapid spread through susceptible hosts especially when these are housed at high densities (for review, see Chapman et al. 2013). Unsurprisingly, resistance to anticoccidial drugs can evolve rapidly under these conditions and there is a continuing need to develop novel therapies (Blake et al. 2011).

More than 1200 species of *Eimeria* are described (Chapman et al. 2013) and virtually all of these are restricted to a single host species. Domestic chickens (*Gallus gallus domesticus*) can be infected by seven *Eimeria* species, each of which colonizes a preferred region of the intestine, causing symptoms of differing severity (Table 1; Shirley et al. 2005). Five species induce gross pathological lesions and four of these are the most important in terms of global disease burden and economic impact (*E. acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella*) (Williams 1998).

Results

Genome sequences of the *Eimeria* species that infect domestic chickens

We generated annotated genome sequences of all seven species of *Eimeria* that infect domestic chickens. For *E. tenella*, a high quality reference genome incorporating annotation-directed manual improvements for targeted regions was produced for the Houghton strain (tier 1) (Supplemental Table S1) as well as Illumina genomic sequencing data for the Wisconsin and Nippon strains. For *E. maxima*, *E. acervulina*, and *E. necatrix*, we produced draft genomes with automated post-assembly improvements (tier 2) (Supplemental Table S1), and for *E. brunetti*, *E. mitis*, and *E. praecox*, we produced

[21]Retired.
[22]Deceased.
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Article published online before print. Article, supplemental material, and publication date are at http://www.genome.org/cgi/doi/10.1101/gr.168955.113. Freely available online through the *Genome Research* Open Access option.

24:1676–1685 Published by Cold Spring Harbor Laboratory Press; ISSN 1088-9051/14; www.genome.org

Table 1. *Eimeria* species biology and genomic *sag* repertoire

Species	Site of development	Disease type	Pathogenicity	*sagA*	*sagB*	*sagC*	Total	Pseudogene fragments
E. praecox H	SI (upper)	M	+	15	0	4	19	20
E. maxima W	SI (mid)	M	+++	35	0	4	39	29
E. acervulina H	SI (upper)	M	++	13	1	2	16	16
E. brunetti H	SI (lower), rectum, caeca	H	++++	61	0	44	105	39
E. mitis H	SI (lower)	M	++	145	0	27	172	128
E. necatrix H	SI (mid), caeca[a]	H	+++++	86	32	1	119	102
E. tenella H	Caeca	H	++++	60	28	1	89	23

Numbers of *sag* genes in each *Eimeria* genome, their breakdown into subfamilies, and the numbers of *sag* pseudogenes (*E. tenella*) or pseudogene fragments (other species). (SI) Small intestine; (M) malabsorptive disease; (H) hemorrhagic disease. Levels of pathogenicity are derived from Long et al. (1976).
[a]Gametogony occurs in the caeca.

draft genomes alone (tier 3) (Supplemental Table S2). The 51.8-Mb *E. tenella* genome assembly corresponds well with a genomic map (described below), suggesting that it accurately reflects the true genome size (Supplemental Table S1). Furthermore the tier 1 and tier 2 assemblies are predicted to be 93%–99% complete with respect to the *T. gondii* genome sequence based on the presence of core eukaryotic genes (Supplemental Table S1; Parra et al. 2007).

To investigate chromosome structures, we used whole-genome mapping to improve contiguity of the tier 1 and 2 genomes and were able to place up to 46% of sequence data onto 15 or 16 optical maps for each genome (Supplemental Table S1), which is close to the haploid chromosome number of 14 (del Cacho et al. 2005). Although few sequence markers were available for each chromosome we were able to map unambiguous identities to six of 15 optical scaffolds in *E. tenella* (Supplemental Table S3).

Phylogeny and synteny between *Eimeria* species

Previous phylogenetic analyses using small numbers of sequences did not fully resolve relationships between *Eimeria* species of the chicken (Ogedengbe et al. 2011). Whole-genome phylogeny shows robust separation of *E. tenella* and *E. necatrix* from the other species, as well as separation of *E. mitis* and *E. brunetti* from *E. praecox*, *E. maxima*, and *E. acervulina* (Fig. 1A). In support of this phylogeny, there is extensive synteny between the genomes of *E. tenella* and *E. necatrix* (i.e., many orthologous genes in the same order across contigs, with only a small number of rearrangements) (Fig. 1B). There is notably less synteny between the genome of *E. tenella* and those of *E. maxima* and *E. acervulina* with much of the chromosome structure rearranged, although presumably retaining the same number of chromosomes. Synteny between the genomes of *E. tenella* and *Toxoplasma gondii* was nonexistent, with no more than three orthologs (ETH_00031645, ETH_00031660, and ETH_00031665) found in the same order.

Eimeria chromosomes display a banded pattern of repeat-poor and repeat-rich regions

Analysis of *E. tenella* chromosome 1 revealed alternating regions of repeat-poor (P) and repeat-rich (R) sequences (Ling et al. 2007). We now find this feature is conserved in all chromosomes of *E. tenella* and across the genomes of all *Eimeria* species examined (Fig. 1B). The short tandem repeat (STR) content of each genome shows a bimodal distribution with a high frequency peak close to zero and a broad, low frequency peak with a mean ~20%–30% (Supplemental Fig. S1A). This confirms a bipartite structure for the genome. Any region of the genome is either repeat-rich (R; which we define as having a repeat density >5%) or essentially repeat-free (P). The precise repeat content differs between species, with *E. tenella* having fewer R regions than other species. Of note, *E. necatrix* is more repeat-rich in regions syntenic with *E. tenella* as well as across the genome generally (Supplemental Fig. S1B). Fifty-three point five percent of *E. tenella* genes were found in repeat-rich regions, suggesting that there is no preference for repeats to occur in gene-poor regions.

Eimeria protein-coding sequences are extremely rich in homopolymeric amino acid repeats (HAARs)

STRs can result in strings of single amino acids within predicted protein sequences. The extent of homopolymeric amino acid repeats (HAARs) is greater in *Eimeria* than in any other organism sequenced to date (Fig. 2A) and the distribution of homopolymer types is quite different from even closely related organisms such as *T. gondii* and *P. falciparum*. The most common STR in *Eimeria* species is the trinucleotide CAG (Fig. 2B), which occurs preferentially in coding sequences (Fig. 2C). CAG can potentially encode alanine (A), glutamine (Q), serine (S), cysteine (C), and leucine (L), but in *E. tenella* repeats are rarely translated as C or L. They are found preferentially as A or Q (Fig. 2D). HAARs of this type, encoding strings of at least seven amino acids, occur in 57% of *E. tenella* genes, with an average of 4.3 copies per gene. We confirmed that repeats are transcribed and translated in *E. tenella*, with the data predicting similar proportions of each HAAR type to that found in the genome (Fig. 2D).

An analysis of gene ontology terms showed that genes containing HAARs did not cluster in any particular functional class. However, those involved in information processing tasks such as translation, chromatin assembly, gene expression, and DNA metabolism had fewer HAARs than expected by chance (Supplemental Table S4). Indeed genes conserved across the eukaryotes had an overall lower than average repeat content (2.5% vs. 4.68% for all *E. tenella* genes). Proteins that are generally considered to be involved in host-parasite interactions such as SAGs, ROP kinases, and MICs had even fewer HAARs on average than those conserved across eukaryotes (0%, 1.88%, and 1.66%, respectively, vs. 2.5%).

We hypothesized that because HAARs are so common in *Eimeria* species, they are unlikely to interfere with protein structure and function. Indeed only 3.2% of *E. tenella* HAARs (687 of 21,191) occur in Pfam domains, which make up 12.4% of *E. tenella* protein sequences. By examining conserved proteins with known 3D structures, we found that serine and glutamine HAARs tend to be insertions in loop or turn regions with medium to high solvent

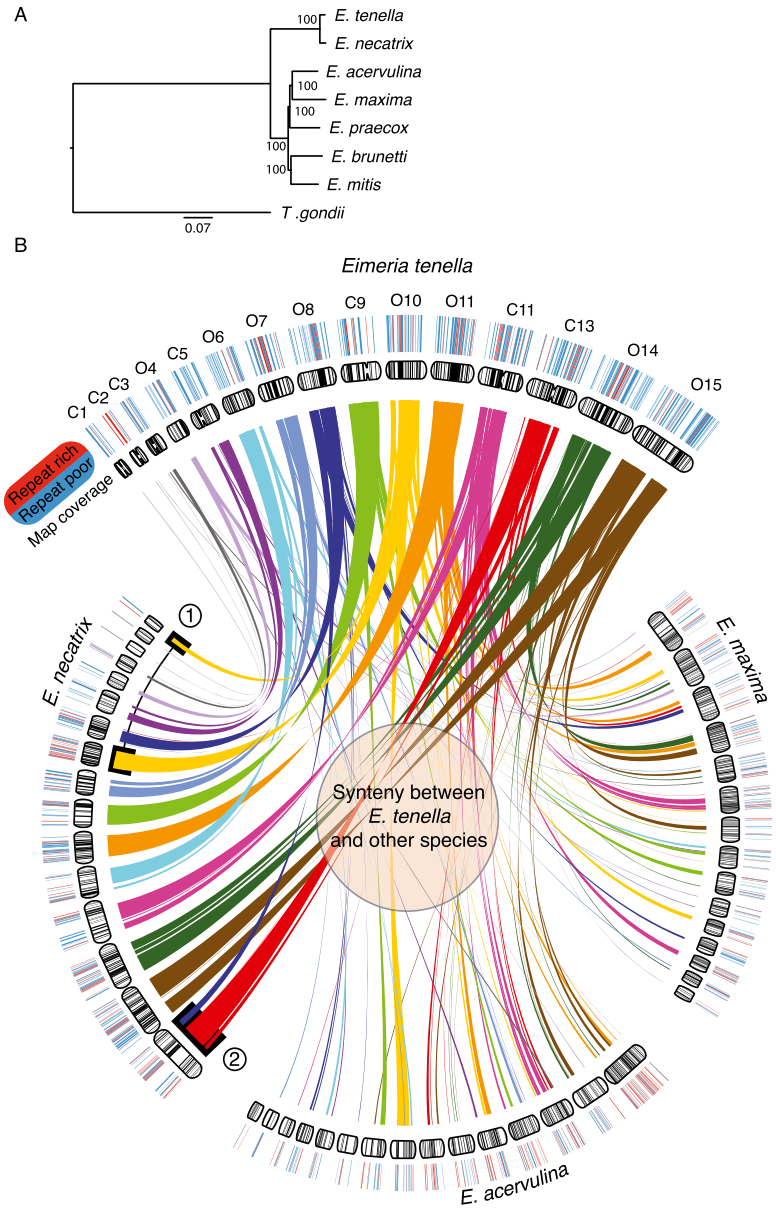

Figure 1. Whole-genome phylogeny and synteny between *Eimeria* species. (*A*) Maximum likelihood phylogeny showing the evolutionary relationships between *Eimeria* species based on alignment of 814 one-to-one orthologs shared with *T. gondii*. The scale is in substitutions per site. (*B*) Genomic scaffolds were placed onto optical maps. Black bands show map coverage. Coverage was noticeably better for *E. tenella* than the tier 2 species. *E. tenella* maps are named as chromosomes (e.g., C1) where it was possible to reliably identify that chromosome, otherwise they are given their optical map numbers (e.g., O4). Each *E. tenella* map has been assigned a color and ribbons highlight syntenic regions in the related genomes. *E. necatrix* is most closely related to *E. tenella* and correspondingly shows the greatest degree of synteny. The clearest exceptions are (1) O10, which is split between two optical contigs in *E. necatrix*, and (2) O8, which is similarly split. Map coverage is lower in *E. acervulina* and *E. maxima* and this gives the impression that there is a great deal of novel sequence in these species. However, this is largely the result of differential representation of the genomes in their respective maps. Each map is annotated with repeat-poor (blue) and repeat-rich (red) regions ≥30 kb. This highlights the barcode-like patterning across the whole of each genome.

accessibility, suggesting they do not affect protein folding (Supplemental Table S5). Alanine HAARs often align to helical regions and may result in very similar local structure (Perutz et al. 2002). Furthermore, homology modeling showed that HAARs tend to be located on the outside of proteins, away from regions involved in domain–domain interactions and active sites (Supplemental Fig. S2).

Comparative genomics of the Coccidia and wider Apicomplexa

Eight thousand, six hundred and three protein-coding genes are predicted in the *E. tenella* assembly, significantly more than the 7286 found in the related coccidian *T. gondii* (Supplemental Table S1), despite *T. gondii* having a nuclear genome that is ~20% (10Mb) larger. By transcriptome sequencing we identified expression of 76% of predicted *E. tenella* genes (6700) across four developmental life stages (unsporulated oocyst, sporulated oocyst, sporozoite, and merozoite). The median sequence identity between *E. tenella* and *T. gondii* orthologous protein sequences was 39.7%, suggesting a large amount of sequence divergence between the two.

We identified several novel *Eimeria*-specific gene families (Supplemental Table S6; Supplemental Data set S1). We found that two of these families (*esf1* and *esf2*) have higher K_a/K_s ratios than other genes (Supplemental Fig. S3; Supplemental Data set S2). This suggests that they may be under diversifying selection and could be important for host–parasite interactions.

The rhoptry organelles of *T. gondii* contain 30–50 kinases and pseudokinases (ROPKs) (Peixoto et al. 2010; Talevich and Kannan 2013), some of which are involved in remodeling the intermediate host cell and protecting the parasite against host defenses (Saeij et al. 2007; Fentress et al. 2010). Recent analysis showed that *E. tenella* has 28 *ropk* genes, including a subfamily not found in *T. gondii* (Talevich and Kannan 2013). We were able to identify orthologs of all these genes in *E. necatrix*; however, there is divergence in the more distantly related *Eimeria* species (Supplemental Table S7; Supplemental Data set S1). The overall protein kinase (PK) complement of *Eimeria* species (63–84 PK) is smaller than that of *T. gondii* (128 PK) (Peixoto et al. 2010) and *Plasmodium* species (85–99 PK) (Ward et al. 2004; Anamika et al. 2005; Miranda-Saavedra et al. 2012). This is not due solely to fewer *ropks* and *fikks* (an apicomplexan family highly expanded in *Plasmodium*) but also a reduction in CMGC kinases (the group which includes cyclin-dependent kinases) (Supplemental Table S7). It is proposed that CMGC kinases have evolved independently within the Apicomplexa to provide specialized functions related to lifecycle transitions (Talevich et al. 2011). Reduction of CMGC kinases in *Eimeria*, which has a simple life cycle and no intermediate host, may be an example of this specialization.

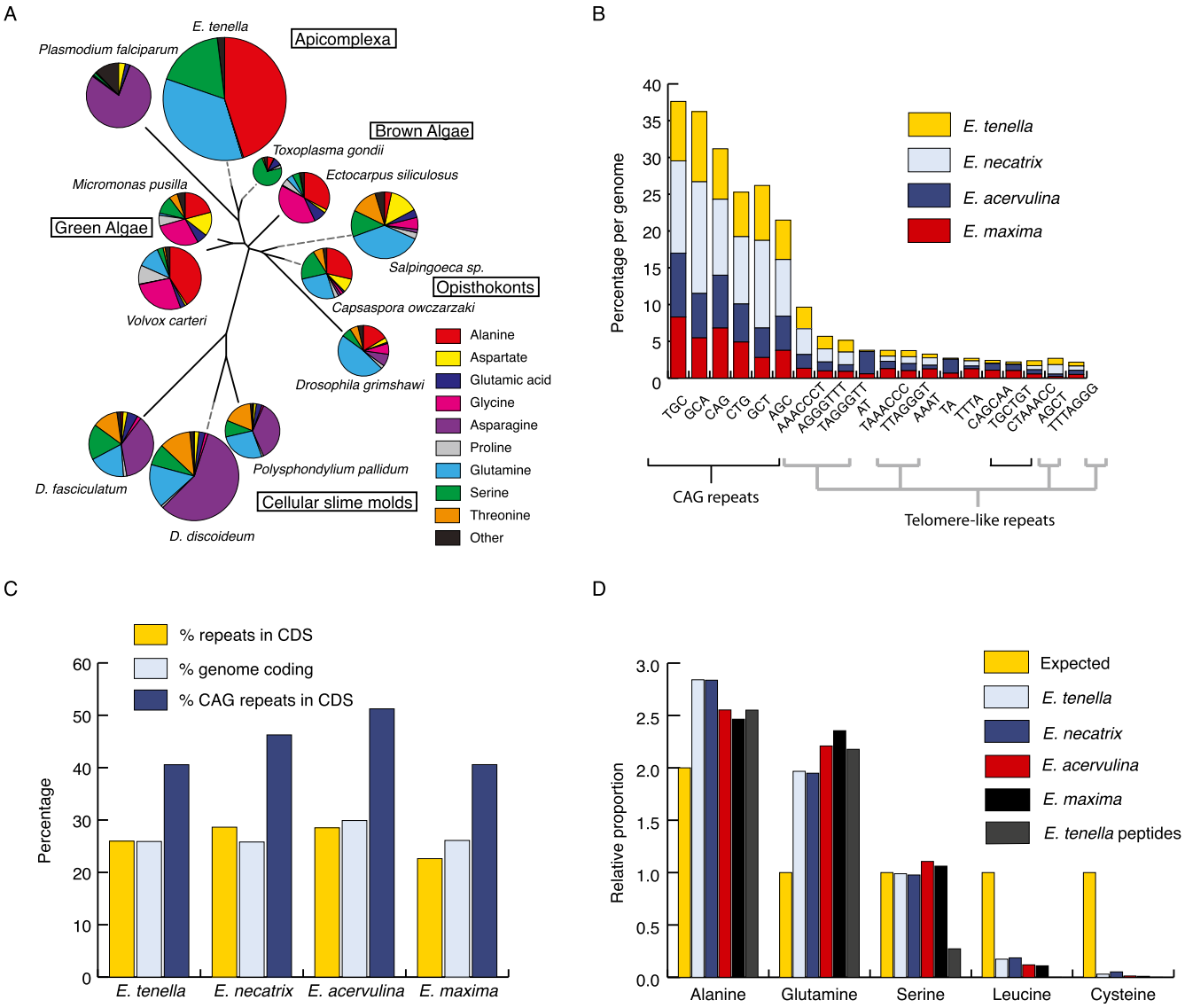

Figure 2. Characterization of HAARs in *Eimeria* protein sequences. (*A*) *Eimeria tenella* has a greater number of HAARs than any other genome sequenced and a distinct distribution of HAAR types compared with other repeat-rich genomes, including *Plasmodium falciparum* and the more closely related and not especially repeat-rich *Toxoplasma*. (*B*) The most common STRs in *Eimeria* genomes are variations on CAG. The second most common are variations on a telomere repeat which we call telomere-like repeats due to their locations throughout the genome. (*C*) CAG repeats occur in protein-coding regions of the genome more than expected. (*D*) CAG repeats can encode strings of one of five amino acids. In *Eimeria* they tend to encode alanine and glutamine more often than expected, serine as often as expected, and leucine and cysteine more rarely than expected. A very similar pattern is observed in a limited selection of *E. tenella* peptides derived from proteomics experiments.

Metabolism is well conserved between *Eimeria* and *Toxoplasma* (Supplemental Fig. S4; Supplemental Data set S3), with the clearest difference being additional enzymes involved in *Eimeria* carbohydrate metabolism. Three of these catalyze reactions in the mannitol cycle, known to be essential for survival of *Eimeria* parasites and not present in other coccidian lineages (Schmatz et al. 1989; Liberator et al. 1998).

The apicoplast is a symbiotic plastid present in most apicomplexans and known to be essential for survival of *T. gondii* (He et al. 2001). Most ancestral plastid genes have moved into the nuclear genome but many of the gene products are post-translationally imported into the apicoplast. The mechanism of import to the apicoplast is poorly understood but two proteins, Tic20 and Tic22, are thought to mediate crossing of the innermost membrane (van Dooren et al. 2008; Lim and McFadden 2010). Genes encoding Tic20 and Tic22 are not found in the *Eimeria* species studied, suggesting either a distinct mechanism for crossing the apicoplast inner membrane or a change in apicoplast function in *Eimeria*.

Apicomplexan genomes have a paucity of common eukaryotic transcription factors (Coulson et al. 2004); instead, the major regulators of stage-specific gene expression are likely to be genes containing DNA-binding domains of the ApiAP2 family (Balaji et al. 2005; Campbell et al. 2010). In *Eimeria* the number of genes containing ApiAP2 domains was found to vary from 44 to 54 (Supplemental Fig. S5; Supplemental Data set S1). We clustered genes containing ApiAP2 DNA-binding domains from apicomplexans and representative outgroups and identified 121 orthologous groups (Supplemental Fig. S6). We found 21 *Eimeria*-specific ApiAP2 groups, 22 further

groups shared by *Eimeria* and other Coccidia, and five pan-apicomplexan clusters (apiap2_og_336, apiap2_og _90, apiap2_og _1428, apiap2_og _546, apiap2_og _456) (Supplemental Data set S1).

We found a positive correlation between the number of ApiAP2 genes and genome size across the Apicomplexa ($r^2 = 0.92$; Pearson) (Supplemental Fig. S5). This suggests that although *Eimeria* has a relatively simple lifecycle compared with some other genera, there is greater complexity in regulating its genome. Thus, we propose that across *Apicomplexa*, it is not the complexity of the developmental lifecycle that determines the complexity of regulation, but the amount of genome to be regulated.

Analysis of chromosome 1 of *E. tenella* identified retrotransposon-like elements (Ling et al. 2007) and we now confirm that these are related to long terminal repeat (LTR) retrotransposons from the group of chromoviruses (Supplemental Fig. S7). Chromoviruses are widespread among eukaryotic genomes but have not previously been identified in apicomplexans (Kordis 2005). With the exception of *eimten1* in the *E. tenella* and *E. necatrix* genomes, the putative transposons are highly fragmented and diverged, indicating very little recent activity (Supplemental Figs. S8, S9; Supplemental Table S8). Retrotransposons cannot have been transferred horizontally from the host, because the chicken genome does not contain chromoviruses (Kordis 2005). Phylogenetic analysis of predicted reverse transcriptases from *Eimeria* and other species did not robustly support a closer relationship with either plant/algal or vertebrate/fungal lineages (Supplemental Fig. S7).

Eimeria-specific surface antigen genes

All apicomplexan genomes examined to date possess gene families encoding antigenic proteins that are expressed on the surface of invasive stages and thought to be important in interaction with the host immune system (Spence et al. 2013). The principal surface antigen gene family in *E. tenella* is *sag*, which encodes single domain, membrane-bound proteins tethered by GPI anchors to the surface of invasive sporozoites and merozoites (Tabares et al. 2004).

In *E. tenella* the majority of *sag* genes are tandemly arrayed in four clusters, each on a different chromosome (Fig. 3A). There are three subfamilies of *sag* genes: *sagA* is common to all species; *sagB* is restricted to *E. tenella* and *E. necatrix*; and *sagC* is restricted to the other species, being most expanded in *E. brunetti* and *E. mitis* (Table 1). All subfamilies encode signal peptides and addition sites for GPI anchors, but the *sagC* extracellular domain contains only four conserved cysteines whereas *sagA* and *sagB* have six. The *sagB* and *sagC* genes each have five exons, suggesting they may be more closely related to each other than to *sagA* genes, which have four exons. The presence of s*agA* genes in all the *Eimeria* species suggests that these provide a core function, while *sagB* and *sagC* genes may provide functions specific to the different clades.

One core function of SAG proteins may be attachment to host cells prior to parasite invasion. In *T. gondii* the *srs* genes play a role in primary attachment and it is known that *E. tenella* SAG1 binds mammalian cells (Jahn et al. 2009). We found that multiple SAGA, but not SAGB proteins, were able to bind cultured cells (Fig. 3B), suggesting that attachment is a potential function of the *sagA* family.

It is of key importance in designing vaccines to understand the array of antigens presented to the host immune system. Using single-cell RT-PCR we found that multiple members of the *sagA* and *sagB* subfamilies were co-expressed in individual sporozoites and merozoites of *E. tenella* (Fig. 3C). Thus the parasite likely presents a complex set of antigens to the host, much like *T. gondii* SRSs, rather than a single one like *Plasmodium falciparum* PfEMP1.

Analysis of stage-specific *sag* expression in populations of clonal *E. tenella* suggests that the expressed repertoire is most complex in second-generation merozoites (Fig. 3D). A small number of *sagA* genes peak in expression at each stage; *sagB* genes all peak in expression in second-generation merozoites, suggesting that they may be particularly important during the later, pathogenic, stages of infection (Fig. 3D).

The total number of *sag* genes varies greatly between species (Table 1). This may simply reflect the overall phylogeny (see Fig. 1A), but it is notable that species that cause the most severe pathologies have higher numbers of *sags*. Thus *E. praecox*, which causes only superficial damage and is widely regarded as the least pathogenic (Allen and Jenkins 2010), has only 19 *sags*, whilst *E. tenella*, *E. necatrix*, and *E. brunetti*, which develop deep in the mucosa causing tissue damage, inflammation, and intestinal hemorrhage (McDonald and Shirley 1987), have 89, 119, and 105 *sags*, respectively. However, *E. mitis*, with the greatest number of *sags* (172), does not fit this pattern; like all species it can impair bird performance and productivity but does not cause gross lesions in the intestine. It is also the case that species which induce potent immunity against reinfection after exposure to small numbers of parasites (*E. maxima*, *E. praecox*, and *E. acervulina*) have the lowest numbers of *sags* whilst those that are least immunogenic (*E. necatrix* and *E. tenella*) have high numbers; however, *E. brunetti* and *E. mitis*, which are of intermediate immunogenicity, do not fit this pattern.

We used remote homology detection to explore the evolutionary origin of the *sag* family. The cysteine-rich secretory protein family (CAP), found in a wide range of eukaryotes (Gibbs et al. 2008), had low but significant sequence similarity to *sags* (Fig. 3E). The most similar CAP-domain containing proteins were those of *T. gondii*, suggesting that *sag* genes are likely derived from CAP-domain containing genes in the common ancestor of *E. tenella* and *T. gondi*, rather than by horizontal transfer from another species such as the host. Recent protein structural evidence shows that the *srs* surface antigen gene family in *T. gondii* is related to a small group of cysteine-rich proteins in *Plasmodium* (Arredondo et al. 2012). These results suggest distinct evolutionary origins for the principle surface antigen gene families of the relatively closely related *T. gondii* and *E. tenella*.

Discussion

Control of pathogens such as *Eimeria* species has been essential for development of modern poultry production and is increasingly important for providing global food security. The availability of genomic resources for the seven *Eimeria* species that infect domestic chickens will underpin development and longevity of new anticoccidial drugs and vaccines.

The most striking feature of the *Eimeria* genomes is the disruption of more than half of all protein coding sequences with HAARs. Across every chromosome of each species we found regions where 20%–30% of the sequence comprised simple tandem repeats, interspersed with relatively repeat-free regions. Although a variety of different simple repeats were found outside of coding regions, those within coding regions were almost always based on runs of the trinucleotide CAG. We examined whether these repeats might have a particular function in the proteome but could find no association with known functional groups and showed that the repeats localized to structurally neutral regions within proteins. We hypothesize that poly-CAG is the most benign and easily evolvable coding repeat and that there is sufficient pressure on maintaining repeat banding to allow for frequent deleterious mutations. The repeat regions might function at the DNA level,

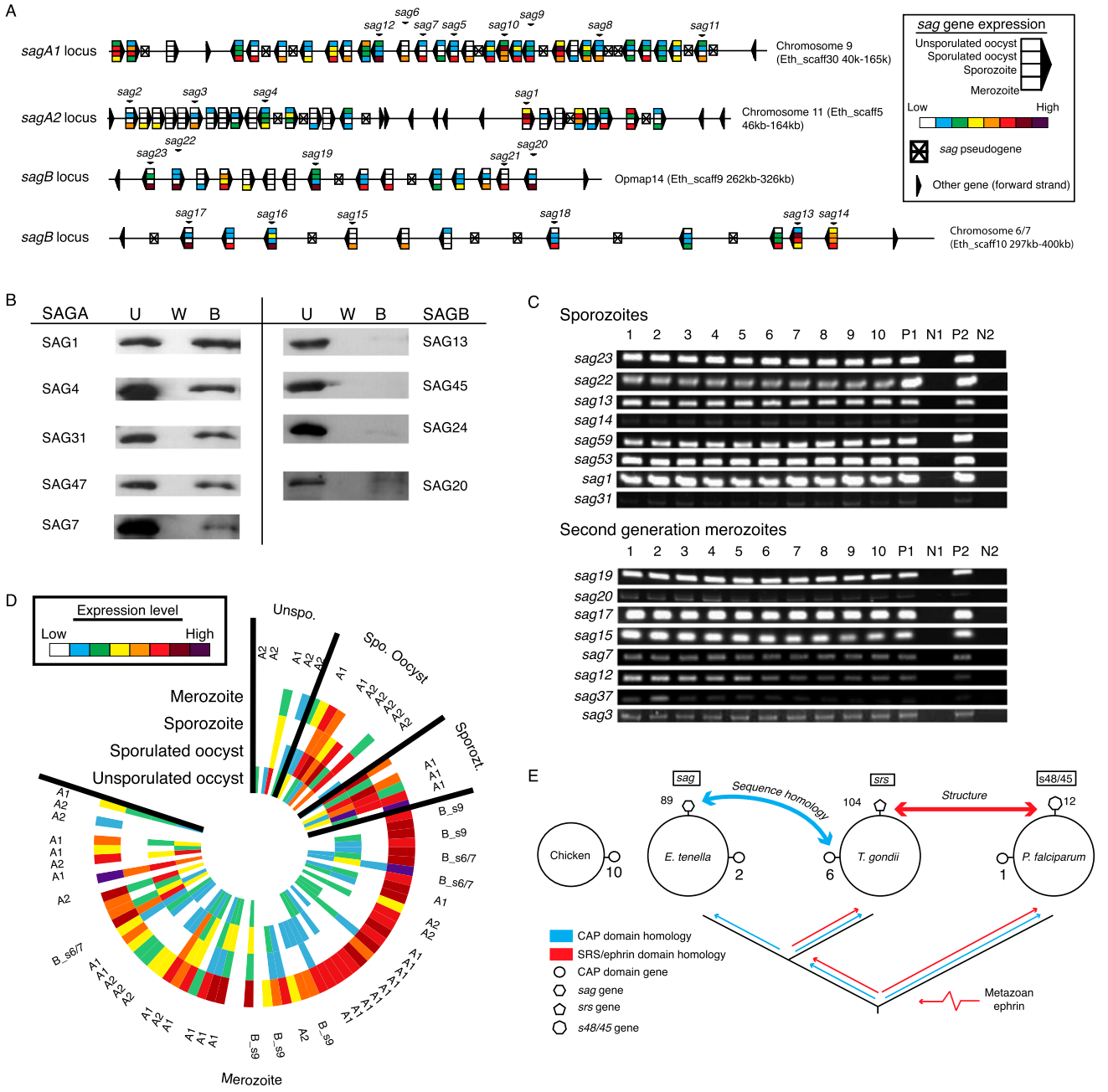

Figure 3. Analysis of the principle family of surface antigens in *Eimeria spp.* (*A*) The four loci of tandemly repeated *sag* genes in *E. tenella* are shown with each *sag* gene represented by bars describing the relative expression levels in four stages of the lifecycle. Gene names used previously in the literature are shown where appropriate. Arrows indicate direction of transcription. (*B*) We found that, of those tested, SAG proteins from subfamily A bound host cells, but those from family B did not. (U) Unwashed; (W) washed; (B) bound. (*C*) Expression of multiple *sag* genes in individual cells of *E. tenella* was detected using RT-PCR. Ten cells were analyzed for each of eight genes in both sporozoites and second-generation merozoites. (Lanes *1–10*) Single-cell multiplex test RT-PCRs. Controls: (lane *P1*) positive, cDNA library multiplex RT-PCR; (lane *N1*) negative, no template multiplex RT-PCR; (lane *P2*) positive, single sporozoite, single target RT-PCR; (lane *N2*) negative, single sporozoite, single target PCR with no RT. (*D*) Expression values for all *E. tenella sag* genes were clustered and those clusters ordered by mean peak expression, showing that most genes peak in the second-generation merozoite. Where appropriate, genes are annotated with their genomic locus as defined in *A*. (*E*) Patterns of homology for *Eimeria sag* and *Toxoplasma srs* genes suggest that while *Toxoplasma* acquired the precursors to its key family of 6-cys surface antigens from a horizontal gene transfer of metazoan ephrin, *Eimeria* has derived them from the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 protein (CAP) family already found in Apicomplexa. Thin, single-headed arrows show phylogenetic paths for CAP- and ephrin-related domains, while bold arrows show the best evidence for the closest relatives of the *sag* and *srs* families. Numbers indicate gene frequencies for each family.

perhaps in tertiary structure or gene regulation, and be selected for functional neutrality at the protein level, as we have observed. This hypothesis could be explored by chromatin immunoprecipitation and chromosome conformation capture studies. An alternative hypothesis is that rapid mutation of coding sequences provides evolutionary advantages, which would suggest selection for re-

peats at the protein level. Although the HAARs we examined appeared to be neutral in their effects on protein structures (presumably deleterious changes are lost in the population), occasional mutations may have been sufficiently beneficial to support the heavy burden. However, genes known to be involved in host–parasite interaction were found to be relatively protected from HAARs, and why repeats should have appeared in a banded pattern across chromosomes is not clear in this scenario.

We have characterized, for the first time, retrotransposon-like elements in an apicomplexan. Disruption or replacement of genes by transposon-mediated transgenesis holds the potential to improve our molecular arsenal for unraveling parasite biology, and also for developing attenuated vaccines. This approach has been attempted in *Eimeria* species using *piggyBAC* transposons; however, rates of insertion of these elements are low and target particular sequences (Su et al. 2012). Random, high-frequency insertions of native transposons would allow high throughput knockout screens, accelerating our understanding of *Eimeria* biology. The retrotransposons identified here are at best partially degraded and it is not clear whether they are actively retrotransposed. However, it opens the possibility that intact retrotransposons might be present in more distantly related *Eimeria* species.

To develop cheap, effective new vaccines for coccidiosis, we must understand how the parasites interact with the host immune system. Key to this may be the *sag* family of genes encoding surface antigens, some of which have been shown in vitro to induce pro-inflammatory cytokine responses (Chow et al. 2011). Whilst some *sags* are shared across all the *Eimeria* species studied, others are clade-specific. We found multiple *sags* to be co-expressed on the surface of infective parasites, suggesting that the avian immune system is presented with a diverse array of related epitopes, which could potentially aggravate inflammation. Parasites that rely on a healthy host for vector transmission are thought to use diverse arrays of related antigens to reduce pathogenicity (Spence et al. 2013) but for *Eimeria*, which has a simple oral-fecal life cycle, the induction of inflammation leading to diarrhea could increase parasite transmission. *Eimeria sag* genes have evolved from the CAP-domain superfamily of cysteine-rich secretory proteins and are unrelated to the major surface antigens of other Apicomplexa. CAP domains are also found on the surface of parasitic helminths where they are proposed to interact with host immune systems (Chalmers and Hoffmann 2012).

Methods

Parasite cultivation

The Houghton (H) strains of *E. acervulina*, *E. brunetti*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*, and the Weybridge (W) strain of *E. maxima*, were used for principle sequencing. The H strains were isolated at the Houghton Poultry Research Station (UK) and are the progeny of single oocyst infections. The *E. maxima* W strain was isolated at the Weybridge Central Veterinary Laboratory from a single oocyst infection. The Wisconsin (Wis) (McDougald and Jeffers 1976) and Nippon-2 (Nt2) *E. tenella* lines were used for comparative analyses. All parasites were propagated in vivo in 3- to 7-wk-old Light Sussex chickens under specific pathogen free (SPF) conditions at the Institute for Animal Health or Royal Veterinary College and purified using established methods (Long et al. 1976).

Summary of genome sequencing and assembly

Genomic DNA was prepared from purified sporulated oocysts. We used Sanger capillary sequencing data previously generated for *E. tenella*, described in Ling et al. (2007) and deposited in the Trace Archive (http://www.ncbi.nlm.nih.gov/Traces; CENTER_PROJECT = "EIMER"). These paired reads had a range of insert sizes to a combined coverage of ~8×. *E. tenella* Illumina GAIIx sequencing libraries were prepared with insert sizes of 300 bp and 3 kbp and either 54 bp or 76 bp paired-end reads with a combined coverage of ~160×. Capillary reads were assembled using ARACHNE v3.2 using default parameters (Batzoglou et al. 2002). IMAGE (Tsai et al. 2010) was used to fill gaps in scaffolds and extend contigs with Illumina reads, running six iterations (three with k-mer = 31 and three with k-mer = 27), mapping with BWA (Li and Durbin 2009). The consensus sequence from the ARACHNE-IMAGE assembly was corrected with Illumina reads using iCORN (Otto et al. 2010). All Illumina reads which did not map to this assembly were assembled using Velvet (Zerbino and Birney 2008) and these contigs were added to the final assembly.

For tier 2 (*E. necatrix* H, *E. maxima* W, and *E. acervulina* H) and tier 3 (*E. mitis* H, *E. praecox* H, and *E. brunetti* H) genomes, 500-bp Illumina TruSeq libraries were prepared and sequenced as 76-bp paired-end reads on an Illumina HiSeq 2000 platform to a depth of 199× theoretical genome coverage for *E. acervulina*, 288× for *E. maxima*, 559× for *E. necatrix*, 143× for *E. brunetti*, 520× for *E. mitis*, and 102× for *E. praecox* as in Kozarewa et al. (2009). Reads were assembled using Velvet (Zerbino and Birney 2008), scaffolding was performed with SSPACE (Boetzer et al. 2011), and gaps were filled using IMAGE (Tsai et al. 2010).

Full methodological details of genome sequencing, assembly, and annotation are provided in the Supplemental Material.

Whole-genome phylogeny

Each of 814 one-to-one ortholog groups shared across the seven *Eimeria* species with *Toxoplasma gondii* ME49 was aligned using MAFFT v7 (Katoh and Standley 2013). Highly variable sites were trimmed using trimal (Capella-Gutierrez et al. 2009) ("automated1" option). The alignments were concatenated using FASconCAT (Kuck and Meusemann 2010) and the resulting alignment used to construct a maximum likelihood phylogenetic tree using RAxML with the model PROTGAMMALG4XF (Stamatakis et al. 2005; Le et al. 2012), bootstrap n = 100. Bootstrap percentage support is shown along the branching nodes. The tree was rooted using *T. gondii* as the outgroup.

Transcriptome sequencing and analysis of *Eimeria tenella*

Unsporulated oocysts (two biological replicates), sporulated oocysts (single replicate), purified sporozoites (single replicate), and second-generation merozoites (single replicate) of the *E. tenella* H strain were selected for transcriptome analysis after harvest and purification as described previously (Novaes et al. 2012). Total RNA was extracted from oocysts using a Qiagen RNeasy kit (Qiagen) and from sporozoites and second-generation merozoites using the Qiagen RNeasy Animal Cells purification protocol as described by the manufacturer. All samples were DNase-treated using the Qiagen RNase-free DNase kit during RNA purification.

Library preparation, sequencing protocols, and data processing were the same as for Reid et al. (2012). For *sag* gene expression clustering in Figure 3D, mean RPKM values were taken for unsporulated oocyst samples and clustering performed using MBCluster.seq with 10 clusters (Si et al. 2014). Clusters were ordered by the stage of peak expression across each cluster. The figure was drawn with Circos (Krzywinski et al. 2009).

Synteny analysis

MCScanX (Wang et al. 2012) was used to determine regions of synteny between pairs of species based on the order of pairwise

orthologs identified using OrthoMCL (Li et al. 2003). Default values were used except for the MATCH_SIZE option, which was set to 3 for comparison of *E. tenella* and *T. gondii*. For other comparisons it was set to 5.

Classification and analysis of gene families

We used several methods to identify and classify metabolic genes, protein kinases, transcription factors, and *Eimeria*-specific gene families. Full methodological details are available in the Supplemental Material.

Retrotransposon analysis

Approaches based on domain content and the presence of long terminal repeats were used to identify and classify retrotransposons. Full methodological details are available in the Supplemental Material.

Single-cell expression of *sag* genes

Single fluorescent *E. tenella* sporozoites and second-generation merozoites (Clark et al. 2008) were sorted into 96-well plates. RNA was reverse-transcribed for a panel of target transcripts by adding gene-specific reverse primers (Supplemental Table S9). Stage-specific multi-cell-derived cDNA or single-cell cDNA with each single-plex primer pair were included as positive controls P1 and P2. No template multiplex and no reverse transcription reactions were included for each assay as negative controls N1 and N2.

MDBK cell binding by SAGs

Confluent monolayers of MDBK cells were blocked with 1% BSA in PBS for 2 h at 4°C, washed three times in PBS, then incubated with recombinant-expressed EtSAG proteins (0.5 mg/mL) for 1 h at 40°C. Monolayers were washed four times with PBS to remove unbound proteins, then cells and bound proteins were solubilized in SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose by electroblotting, and probed with rabbit hyperimmune sera raised to recombinant SAG proteins.

Classification of repeat regions

We used Tandem Repeat Finder (Benson 1999) to identify STRs in genomic sequences. It was run with the following parameters: 2, 1000, 1000, 80, 10, 25, 1000, as were used in Ling et al. (2007) and processed using TRAP v1.1 (Sobreira et al. 2006), run with default parameters. We defined a repeat density of $\geq$0.05 over a window of 1 kb as defining a repeat-rich region based on the distribution of repeat density across *E. tenella*. Scaffolds shorter than 5 kb were not included in the analysis.

We used SEG (Wootton and Federhen 1996) with parameters 7, 0, 0, –l, to identify HAARs of length $\geq$7. Repeats of "X," the symbol used when the amino acid is unknown, and repeats which contained stop codons were removed from the SEG output file. To determine the HAAR content of protein sequences in other organisms, we ran SEG on all complete proteomes from UniProt (~2000 species, ~10-m sequences). We then ranked each species by the median number of repeats per sequence. We took the top 10, excluding viruses, and also *T. gondii* for comparison.

Full methodological details of protein structural and proteomic analyses of HAARs are available in the Supplemental Material.

Data access

Sequencing reads and assemblies for each genome have been submitted to the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena/): *E. tenella* Houghton, *E. maxima* Weybridge, *E. acervulina* Houghton, *E necatrix* Houghton, *E. mitis* Houghton, *E. brunetti* Houghton, and *E. praecox* Houghton (PRJEB4918). Sequencing reads for *E. tenella* Nippon and *E. tenella* Wisconsin have been submitted to ENA (PRJEB4009). Transcriptome sequences for *E. tenella* Houghton have also been submitted to ENA (ERP001847) and ArrayExpress (https://www.ebi.ac.uk/arrayexpress/; E-ERAD-109).

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Acknowledgments

The work was funded by BBSRC grants S17413 and S19705/6, Wellcome Trust core funding to Wellcome Trust Sanger Institute (WTSI), and faculty baseline funding from the King Abdullah University of Science and Technology (KAUST). We thank Mike Quail, Karen Mungall, Carol Churcher, and members of the core DNA pipelines for sequencing at WTSI and Bioscience core laboratories for sequencing operations at KAUST. We acknowledge Martin Aslett from WTSI for data submission and Dora Harvey and Fionnadh Carroll from the Institute for Animal Health for technical assistance. K.-L.W. would like to acknowledge funding from the Ministry of Science, Technology and Innovation, Malaysia (Project No. 07-05-16-MGI-GMB10) and the Universiti Kebangsaan Malaysia (Project No. DIP-2012-21). J.P. and S.S.H. were funded by the Canadian Institute for Health Research (CIHR #MOP84556).

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Received October 29, 2013; accepted in revised form July 8, 2014.