The Genome of Model Malaria Parasites, and Comparative Genomics

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Abstract
The field of comparative genomics of malaria parasites has recently come of age with the completion of the whole genome sequences of the human malaria parasite *Plasmodium falciparum* and a rodent malaria model, *Plasmodium yoelii yoelii*. With several other genome sequencing projects of different model and human malaria parasite species underway, comparing genomes from multiple species has necessitated the development of improved informatics tools and analyses. Results from initial comparative analyses reveal striking conservation of gene synteny between malaria species within conserved chromosome cores, in contrast to reduced homology within subtelomeric regions, in line with previous findings on a smaller scale. Genes that elicit a host immune response are frequently found to be species-specific, although a large variant multigene family is common to many rodent malaria species and *Plasmodium vivax.* Sequence alignment of syntenic regions from multiple species has revealed the similarity between species in coding regions to be high relative to non-coding regions, and phylogenetic footprinting studies promise to reveal conserved motifs in the latter. Comparison of non-synonymous substitution rates between orthologous genes is proving a powerful technique for identifying genes under selection pressure, and may be useful for vaccine design. This is a stimulating time for comparative genomics of model and human malaria parasites, which promises to produce useful results for the development of antimalarial drugs and vaccines.

Introduction

Model malaria parasites have proven invaluable in the study of the human form of the disease, where host specificity represents a major constraint for laboratory-based experimentation. Before the development of *in vitro* cultivation of *Plasmodium falciparum,* animal models of malaria were widely used and provided researchers with a means to develop a better understanding of the biology of the parasite and its interactions with the mammalian host and vector (Waters, 2002; Waters and Janse, 2004). Their role in providing biological insight continues today, since certain aspects of malaria pathology and biology, for example invasion of hepatocytes by sporozoites (Mota *et al.*, 2001), cannot be studied without the use of an animal model system. In many instances they also provide the only source of biological material for several life-cycle stages, such as ookinetes and zygotes (Janse *et al.*, 1995). Moreover, their usefulness in functional characterization of genes through gene knock-out and modification studies is well established (de Koning-Ward *et al.*, 2000). Three groups of model systems can be identified: (1) simian malaria species that naturally parasitize non-human primates, for example the *Plasmodium knowlesi*/macaque monkey model system; (2) species of bird malaria that infect domestic fowl, for example the *Plasmodium gallinaceum*/domestic chicken model system; and (3) species of African thicket rat parasite that have been adapted for growth in laboratory rodents. The latter group, consisting of four species *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium vinckei* and *Plasmodium yoelii*, have been the most widely used as models for the study of *P. falciparum* malaria, primarily due to the ease of handling and maintaining rats and mice in the laboratory. In terms of evolutionary relatedness, studies involving the comparison of homologous genes from different *Plasmodium* species have shown that the genus is comprised of several deep branches. The four human malaria species *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* form separate clades, with *P. vivax* showing distinct clustering with monkey malaria parasites such as *P. knowlesi*, and *P. falciparum* more closely associated with avian malaria species (Escalante *et al.*, 1994; Waters *et al.*, 1991). The rodent malaria species also form a distinct clade.

The notion of a universal model for the study of all human malaria species has been shown to be untenable, and instead, a view of model species selection based upon the complement of genes within the model that best fit the phenotypic trait under study, is more appropriate. With the completion of the *P. falciparum* genome sequencing project, undertaken by an international consortium of sequencing centers and malaria researchers, additional genome sequencing projects have started to generate substantial information from other model and human *Plasmodium* species, enabling the full gene complement to be identified within each species. Thus, comparative analysis of genome data from multiple malaria species is now a tangible prospect.

A current list of malaria parasite genome initiatives is given in Table 1. The genomes of two species have been sequenced and published to date, the complete finished sequence of *P. falciparum* (Gardner *et al.*, 2002), and the partial sequence of one of the four species of rodent malaria parasites, *Plasmodium yoelii yoelii* (Carlton *et al.*, 2002). Other current sequencing projects include partial shotgun coverage of the monkey malaria parasite *P. knowlesi* and two more rodent malaria parasite species

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Table 1. Malaria parasite genome, transcriptome and proteome projects.

Species and strain, line or clone	Genome project goal (current status)	Sequencing Center	[a]ESTs in GenBank dbEST	[b]GSSs in GenBank dbGSS	Microarray projects	Proteomic projects
P. falciparum 3D7 clone	Finished genome (complete and published)	TIGR, Sanger Institute, Stanford University	17,939	1,782	cDNA arrays gDNA arrays oligo arrays SAGE	asexual stages food vacuole sporozoite gametocyte gamete
P. falciparum patient isolate	Partial to 3X (underway)	Sanger Institute				-
P. vivax Salvador I strain	Full shotgun and closure to 8X (gap closure)	TIGR	-	10,682	-	-
P. yoelii yoelii 17XNL clone	Partial to 5X (complete and published)	TIGR	15,562	-	cDNA arrays oligo arrays	asexual stages sporozoite gametocyte ookinete
P. chabaudi chabaudi AS clone	Partial to 3X (complete)	Sanger Institute	-	-	-	-
P. berghei ANKA clone	Partial to 3X (complete)	Sanger Institute	5,329	5,476	gDNA arrays	asexual stages sporozoite gametocyte ookinete oocyst
P. knowlesi H	Partial to 5X (complete)	Sanger Institute	-	-	-	-
P. reichenowi Oscar	Partial to 3X (1X)	Sanger Institute	-	-	-	-
P. gallinaceum A	Partial to 3X (underway)	Sanger Institute	-	-	-	-

[a] ESTs: expressed sequence tags from cDNA libraries
[b] GSSs: genome survey sequences from mung bean nuclease-digested gDNA libraries
Data may be accessed through PlasmoDB at http://plasmodb.org (Bahl et al., 2003)

P. berghei and *P. chabaudi chabaudi*, and the finished genome sequence of a second human malaria parasite, *Plasmodium vivax* (Carlton, 2003), with publications describing the annotation and comparative analysis of the genomes expected before the close of 2004 (definitions of genomic terms used throughout this review can be found in Box 1). All sequence data are being released by the sequencing centers to researchers in advance of final publication so that biological experimentation can be 'jump-started'. This has proven highly successful in the case of prior release of the *P. falciparum* genome sequence data, resulting in identification of parasite-specific pathways which may represent unique targets for intervention strategies (see for example Jomaa *et al.*, 1999), while acknowledging the perogative of the sequencing centers to publish a whole genome analysis of the final data.

Comparative analyses of genome data, or 'comparative genomics', encompasses several areas of research. Prior to the production of large-scale genome sequencing data, comparative gene mapping studies showed that relative gene location and order can be conserved over large regions of chromosomes of different species (Graves, 1998). This area of research established criteria for defining homologies between genes of different species, which are still adhered to today (Box 1). With the advent of computational biology, algorithms such as the BLAST series for pairwise sequence alignment (Altschul *et al.*, 1990) and the development of the International Nucleotide Sequence Database, comprising DDBJ, EMBL and GenBank, tools were available for comparative analysis of nucleotide and protein sequence data from different species *in silico*. With the arrival of the genomics and bioinformatics revolution, comparative genomics has scaled up and whole genome comparisons are now used to describe relative genome composition, genome organization, identify orthologous and paralogous genes, classify species-specific genes, and chart the evolution of the organisms being compared, in all three domains of life: bacterial (Fraser *et al.*, 2000), archaeal (Nelson *et al.*, 2000) and eukaryotic (Rubin *et al.*, 2000).

Comparative genomics of malaria parasite genomes is still a science in its infancy. This review will focus primarily on the rodent models of malaria and comparative genomic studies with the human malaria species *P. falciparum*, since these are the most advanced. A brief, general background concerning the *Plasmodium* genome and a description of published studies in comparative genomics are given, but since much of this has been recently reviewed (van Lin *et al.*, 2000; Waters,

Box 1. Glossary of Genome Sequencing and Comparative Genomics Terms

Genome Sequencing Terms	
Raw sequence:	Unassembled sequence reads produced from sequencing of inserts from individual recombinant clones of a genomic DNA library.
Finished sequence:	Complete sequence of a genome with no gaps and an accuracy of > 99.9%.
Genome coverage:	Average number of times a nucleotide is represented by a high-quality base in random raw sequence.
Full shotgun coverage:	Genome coverage in random raw sequence required to produce finished sequence, usually 8-10 fold ('8-10X').
Partial shotgun coverage:	Typically 3-6X random coverage of a genome which produces sequence data of sufficient quality to enable gene identification but which is not sufficient to produce a finished genome sequence
Paired reads:	Sequence reads determined from both ends of a cloned insert in a recombinant clone.
Contig:	Contiguous DNA sequence produced from joining overlapping raw sequence reads.
Singleton:	Single sequence read that cannot be joined ('assembled') into a contig.
Scaffold:	A group of ordered and orientated contigs known to be physically linked to each other by paired read information.
EST:	Expressed sequence tag generated by sequencing one end of a recombinant clone from a cDNA library.
GSS:	Genome survey sequence generated by sequencing one end of a recombinant clone from a genomic DNA library.
SNP:	Single nucleotide polymorphism, i.e a single nucleotide position for which two or more alternative alleles are present at a certain frequency.
ORF:	Open reading frame, stretches of codons in the same reading frame uninterrupted by STOP codons and calculated from a six-frame translation of DNA sequence.
Comparative Genomics Terms	
Homologs:	Genes related to each other by descent from a common ancestral DNA sequence.
Orthologs:	Homologous genes generated by speciation, i.e related to each other by vertical descent.
Paralogs:	Homologous genes generated by duplication, i.e related to each other by horizontal descent.
Conserved synteny:	Three or more genes located on the same chromosome in different species regardless of gene order.
Conserved linkage:	A group of genes conserved in synteny and order between species.

2002), a greater emphasis will be placed on more recent developments and future directions for research.

The Nuclear Genome and Gene Complement of Malaria Parasites

What does the nuclear genome of a typical malaria parasite look like? By taking data from a number of genome projects both partial and finished, it is now possible to create a generalized view (Table 2A). The haploid genome has a standard size of approximately 22-26 Mb (Carlton *et al.*, 2002; Gardner *et al.*, 2002), distributed among 14 linear chromosomes that range in size from 500 kb to over 3 Mb (Carlton *et al.*, 1999; Janse *et al.*, 1994; Kemp *et al.*, 1987). Note: Karyotype data is not available for all *Plasmodium* species, however it is unlikely that any species deviates significantly from this number. Genome composition varies from species to species, and is not host lineage-specific. For example, the (A+T) genome composition of *P. falciparum* is 81% (Gardner *et al.*, 2002) compared to 62% in *P. vivax* (Carlton, 2003). The rodent malaria species have similarly high (A+T)-rich genomes compared with *P. falciparum*, whereas *P. knowlesi* and *P. vivax* are less biased. The genomes of some species have an additional higher order structuring, in that sections of the genome are compartmentalized into discrete regions

or 'isochores' of differing (A+T) content (McCutchan *et al.*, 1984). An example is the simian malaria parasite *Plasmodium cynomolgi* in which protein coding genes have been localized to (G+C)-rich isochores, whereas chromosome ends containing the telomeres appear to be located in (A+T)-rich isochores (McCutchan *et al.*, 1988). Evidence from the complete sequence of two *P. vivax* YACs, one containing a telomeric chromosome segment (del Portillo *et al.*, 2001), and the other a more central chromosome region (Tchavtchitch *et al.*, 2001), supports a similar organization of the *P. vivax* genome. In contrast, *P. falciparum* has a uniform genome composition, with the exception of short regions of >97% (A+T) on each chromosome which most likely contain the centromeres (Hall *et al.*, 2002), and the bias exhibited between coding and non-coding regions (described below). It is tempting to speculate that isochores may encode genes that mediate phenomena specific to the pathophysiology of the species that harbour them (McCutchan *et al.*, 1984), but evidence for this has yet to emerge.

Each *Plasmodium* species appears to have 5,000-6,000 predicted genes per genome (Buckee, 2002; Carlton *et al.*, 2002; Gardner *et al.*, 2002). Of these, 60% represent orthologous genes between the species, as determined by reciprocal best-match *BLAST* analysis (Buckee, 2002; Carlton *et al.*, 2002). Many of the genes unique to each species are located within subtelomeric regions, and many are known to code for immunodominant antigens. The difference in gene number between species is due to (a) gene expansion/contraction in different lineages, for example the *pyst-a* gene family which has more than 150 members in *P. y. yoelii* but only one copy in *P. falciparum* (Table 2A); and (b) the presence of a large variant gene family in some *Plasmodium* species, predicted to be involved in antigenic variation. The family was first described in *P. vivax* [the *vir* family (del Portillo *et al.*, 2001)], and latterly in *P. yoelii* [the *yir* family (Carlton *et al.*, 2002)], *P. berghei* (the *bir* family) and *P. chabaudi* [the *cir* family (Janssen *et al.*, 2002)], and *P. knowlesi* [the *kir* family (Buckee, 2002)]. True homologs of this family so far have not been identified in *P. falciparum*, which contains other gene families involved in antigenic variation and evasion of immune responses [the *var*, *rif*, *clag* and *stevor* gene families (Craig *et al.*, 2001)]. In *P. knowlesi*, the *SICAvar* gene family has also been described (al-Khedery *et al.*, 1999) which is expressed on the surface of infected erythrocytes and is implicated in antigenic variation in this species. No significant homology exists between the *var* and *SICAvar* genes. As a cautionary note however, discrepancies in the number of predicted genes between species may also reflect the incomplete nature of partial genome data, which can exacerbate the problems associated with accurate gene prediction.

A comparison of the *P. falciparum* and *P. y. yoelii* coding and non-coding regions (Table 2B), suggests that different *Plasmodium* species exhibit similar characteristics for these regions (Carlton *et al.*, 2002). For example, coding regions of the genome have a lower (A+T) content (76%) than non-coding regions (80-87%), and a similar percentage of genes contain introns (54%). The main exception appears to be the mean length of genes, which in *P. falciparum* is almost twice the size of the gene length in *P. y. yoelii*, and also larger than the mean length of genes in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Gardner *et al.*, 2002), both lower eukaryotes. The explanation for increased gene length in *P. falciparum* is at present not known.

Besides gene families involved in antigenic variation, comparative analysis of several other nuclear gene families in different *Plasmodium* species is ongoing. For example, members of the *P48/45* gene super family have been identified in *P. falciparum*, *P. berghei*, *P. vivax* and *P. yoelii* (Thompson *et al.*, 2001). This is a large conserved family of proteins expressed during the sexual stages of which there are ten members in *P. falciparum* (J. Thompson, pers. comm), and it is likely that a similar number will be found in the other species. Given the stage-specific expression and role in the development of transmission blocking vaccines of *P48/45*, rodent model orthologs of the family are proving to be immensely valuable in functional analyses of the genes, for example by gene knock-out (van Dijk *et al.*, 2001). Members of the *Py235* multi-gene family, first identified in *P. yoelii* as exhibiting a novel form of clonal antigenic variation whereby each merozoite from a parental schizont has the propensity to express a different Py235 protein (Preiser *et al.*, 2002), have been identified in *P. falciparum* and *P. vivax* (Khan *et al.*, 2001). Examination of their role in merozoite attachment and invasion of specific erythrocytes is proving to be of value for the determination of the mechanism of erythrocyte invasion in different species, such as *P. vivax*, which is restricted to growth in reticulocytes positive for the Duffy blood group antigen complex. Another group of genes involved in *Plasmodium* merozoite invasion and specific recognition of host cell receptors is the *ebl* gene family, which contains six members in *P. falciparum*: *baebl*, *eba-175*, *ebl-1*, *jesebl*, *maebl* and *pebl*, and the *P. vivax* and *P. knowlesi* Duffy-binding proteins (Adams *et al.*, 2001). The *Plasmodium ebl* genes are single copy, have a multi-exon structure encoding distinct functional domains, and conserved exon–intron splice junctions. Gene duplication has been found to be a common characteristic of the family, providing the molecular basis for the development of alternative invasion pathways. Cross-species analysis of the conserved cysteine-rich domains in members of the gene family has identified certain of the genes as having ancient origins which predate the speciation of *Plasmodium* (Michon *et al.*, 2002).

Comparative Gene Expression Studies

Table 1 lists gene and protein expression data being generated for different life-stages of various *Plasmodium* species. Large-scale sequencing projects have generated a substantial number of ESTs and full-length cDNA sequences from *P. falciparum* (Chakrabarti *et al.*, 1994; Watanabe *et al.*, 2002), *P. berghei* (Carlton *et al.*, 2001b; Matuschewski *et al.*, 2002) and *P. y. yoelii* (Kappe *et al.*, 2001), as well as several thousand mung bean nuclease GSSs from *P. vivax*, *P. falciparum* and *P. berghei* (Carlton *et al.*, 2002; Reddy *et al.*, 1993), enabling some preliminary comparative analyses of the transcriptome and proteome of malaria parasites. In

Table 2. *Plasmodium* genome characteristics

A. Comparison of general genome characteristics from six *Plasmodium* genome datasets.

	P. falciparum	***P. knowlesi***	***P. y. yoelii***	***P. c. chabaudi***	***P. berghei***	***P. vivax***
Genome size (Mb)	22.9	23.0	23.1	~23-24 [b]	~23-24 [b]	25.8
No. chromosomes	14	14	14	14	14	14
% (A+T)	80.6	61.9	77.4	75.6	76.1	62.4
Isochore structure	Absent	Absent	Absent	Absent	Absent	Present
No. genes	5,268	5,281	5,878 [a]	ND	ND	ND
Copy no. of largest gene families	149 *rif* 59 *var* 28 *stevor*	194 SICA*var*[a] 36 hypothetical1[a] 33 *kir*[a]	~800 *yir*[a] ~168 *pyst-a*[a] ~57 *pyst-b*[a]	ND	ND	600-1000 *vir*
Centromeres	Functionally uncharacter-ized	ND	ND	ND	ND	ND
Telomeric repeat	AACCCTA	AACCCTA	AACCCTG	AACCCT(G/A)	AACCCT(G/A)	AACCCT(G/A)
Complex subtelomeric repeats	Identified	Identified	Limited	Identified	Identified	Absent

[a] Likely to be an over-estimate due to inclusion of partial genes;
[b] Determined from karyotype data; ND: not determined.

B. Comparison of *Plasmodium* coding and non-coding regions.

Characteristic	***P. falciparum***	***P. y. yoelii***
No. predicted genes	5,268	5,878
Mean gene length (bp)	2,283	1,298
Gene density (bp per gene)	4,338	2,566
Percent coding	52.6	50.6
Genes with introns (%)	53.9	54.2
Mean no. exons per gene	2.4	2.0
Exon (G+C) content (%)	23.7	24.8
Exon mean length (bp)	949	641
Intron (G+C) content (%)	13.5	21.1
Intron mean length (bp)	179	209
Intergenic (G+C) content (%)	13.6	20.7
Intergenic mean length (bp)	1,694	859

one study, clustering algorithms were used to assemble the data and to create several thousand concensus sequences which were compared between *P. falciparum*, *P. berghei* and *P. vivax* (Carlton *et al.*, 2001b). This comparison of partial data identified many protein motifs and signatures as being conserved between the species. Comparison of the Gene Ontology terms [GO terms represent a vocabulary designed to describe all known genes (Ashburner *et al.*, 2000)] assigned to proteins of each species showed similar numbers of proteins in each class for each species, with the exception of the Cell Process and Defense and Immunity classes. This finding was later confirmed by whole proteome comparative analysis of *P. falciparum* and *P. y. yoelii* (Carlton *et al.*, 2002), and reflects the non-homologous Nature of the proteins involved in antigenic variation and evasion of immune responses in *Plasmodium* species. In another study, comparative analysis of genes expressed in salivary gland sporzoites versus those expressed in oocyst sporozoites identified genes that were upregulated

in the former, signifying possible developmental changes in the infectious transmission stage of *Plasmodium* (Matuschewski *et al.*, 2002).

Several microarray analyses of gene expression of whole *P. falciparum* chromosomes (Le Roch *et al.*, 2002) and the complete genome at different developmental stages (Ben Mamoun *et al.*, 2001; Hayward *et al.*, 2000) have been published, as well as serial analysis of gene expression (SAGE) studies (Patankar *et al.*, 2001). The latter study purported to find a significant number of antisense messages in asexual and sexual stages, the first time this has been reported in species of *Plasmodium*. Microarrays of other rodent model species are also in progress (M. Karras and A. Waters, unpublished), with the specific aim of comparing results to the *P. falciparum* expression studies, and enabling a transcriptional profile of orthologous *Plasmodium* genes to be created. Microarays of the mosquito vector have been constructed too, and pilot studies undertaken to determine mosquito genes induced through infection with *P. berghei* (Dimopoulos *et al.*, 2002).

Two large-scale, high-throughput mass spectrometric analyses of *P. falciparum* proteins from sporozoite, merozoite, trophozoite, gametocyte and gamete stages were recently published (Florens *et al.*, 2002; Lasonder *et al.*, 2002), and a smaller analysis of the proteins in sporozoite and gametocyte stages of *P. y. yoelii* (Carlton *et al.*, 2002). These datasets provide validation of gene predictions in both species (52% of predicted *P. falciparum* genes were confirmed by proteomic data). A comparative analysis between these and other ongoing *Plasmodium* proteome projects is underway (D. Raine, L. Florens, R. Sinden and J. Yates, unpublished). Finally, data from a number of transcriptome and proteome projects, and mapping of the expression data to the genome sequence, will facilitate a thorough investigation of the phenomenon of “coordinated gene expression clustering”, as shown to exist in certain eukaryotes (Blumenthal *et al.*, 2002; Caron *et al.*, 2001; Cohen *et al.*, 2000). Gene clustering can be defined in a number of different ways (Carlton, 1999), depending upon whether the genes under study are functionally related, polycistronically transcribed, expressed in the same pathway, or paralogous gene copies generated by gene duplication events. Preliminary evidence exists for some undefined level of synchronized gene expression (Florens *et al.*, 2002), but to what extent and what consequence remains to be determined.

Chromosome Structure, Comparative Mapping and Gene Synteny Studies

Several features of chromosome structure appear to be well conserved in all *Plasmodium* species. All possess telomeres consisting of degenerate, canonical, tandem repeats, the most common motif being AACCCT(A/G) (Scherf *et al.*, 2001). The mean length of the telomeric array (~800 to ~6700 bp) varies from species to species, although it remains remarkably constant within species (Figueiredo *et al.*, 2002). Subtelomeric regions of *Plasmodium* chromosomes consist of a variable number of species-specific repeats that extend 10-40 kb towards the internal part of chromosomes, and which have extensive large-scale similarity between chromosomes, indicative of intra-chromosomal exchange (Carlton *et al.*, 2002; Gardner *et al.*, 2002). Low restriction maps and high-resolution YAC contig maps, in conjunction with the *P. falciparum* and *P. y. yoelii* finished sequence data, have now established that species-specific gene families coding for immunodominant antigens and proteins known to be involved in antigenic variation are predominantly found within these regions, whereas conserved ‘housekeeping’ genes are located within central chromosome regions. Thus *Plasmodium* chromosomes consist of a central conserved core flanked at each end by less conserved regions containing antigen genes. This chromosomal organization has been confirmed at the genomic level by construction of a SNP map of *P. falciparum* chromosome 2 from several parasite isolates using an oligonucleotide array (Volkman *et al.*, 2002). Recently, *P. falciparum* chromosome ends were shown to cluster at the periphery of the nucleus, facilitating ectopic recombination among heterologous subtelomeric chromosome regions and thus providing a mechanism for the generation of different repertoires of antigen genes (Freitas-Junior *et al.*, 2000). Whether this represents a common mechanism shared by other *Plasmodium* species remains to be seen, but it is interesting to note the shared features of chromosome organization between species which would facilitate this.

The chromosomes of *P. falciparum* (Kemp *et al.*, 1985; van der Ploeg *et al.*, 1985), *P. vivax* (Langsley *et al.*, 1988) and rodent malaria species (Janse, 1993) are known to vary considerably in length. Such ‘chromosome size polymorphisms’ are found to occur in field isolates, most likely as a result of unequal recombination between homologous chromosomes of different parasite clones during meiosis, but also by gene amplification, and deletion and insertion of repeat sequences. *P. falciparum* chromosomes are also found to vary in size during *in vitro* culture, due to chromosome breakage followed by healing of the blunt end by the addition of telomeric repeats (Bottius *et al.*, 1998). Most of these large-scale chromosomal rearrangements affect non-coding repeat sequences in the subtelomeric regions, since the conserved core of the chromosome appears less prone to rearrangement. An exception are the genome rearrangements that occur in parasites under selective pressure, which have caused changes in ploidy as well as ‘amplicons’ containing copies of the same gene in tandem (Carlton *et al.*, 2001a). Thus, chromosomal rearrangements in *Plasmodium* are important for the evolution of the genome, although to what extent this occurs in natural populations of the parasite remains to be determined.

Given the range and diversity of karyotypes seen among different species, a surprising result of chromosome mapping experiments has been the high degree of conservation of gene synteny between *Plasmodium* species. Initial studies involving mapping of conserved genes to separations of *Plasmodium* chromosomes showed that gene location (conserved synteny) and gene order (conserved linkage) are preserved over large regions between all four species of rodent malaria (Janse *et al.*, 1994), between species of rodent malaria and *P. falciparum* (Carlton *et al.*, 1998), and between all four human malaria species (Carlton *et*

al., 1999). These studies have now been extended and show that even exon/intron boundaries and the fine-scale organization of genes can be conserved between species (Tchavtchitch *et al.*, 2001; van Lin *et al.*, 2001; Vinkenoog *et al.*, 1995). The degree of conservation of synteny is greatest when comparing genomes of more closely related species. The rodent malaria parasites, for example, show conservation of whole chromosome synteny (Janse *et al.*, 1994), whereas synteny is reduced to the level of conservation of large chromosomal blocks between *P. falciparum* and the rodent malaria species (Carlton *et al.*, 1998).

The initial comparative mapping studies of *Plasmodium* species described above involved hybridization of a limited number of conserved genes to chromosome separations, and the construction of partial genome synteny maps. With the advent of genome technology and bioinformatics, and the availability of large *Plasmodium* genome datasets, it is now possible to use computational methods for whole genome comparative analyses, as described below.

Computational Algorithms for Cross-species Comparisons

To some extent, the availability of sequence data from a number of species has outpaced the computational and experimental methods used to compare and decode the information within the data. Whole genome shotgun sequencing has progressed so much as to be a high-throughput science, however, the computational and analytical software to analyze the data coming from the pipeline has not developed in a similar fashion. Comparative genomics tools are being designed to specifically address this problem (Frazer *et al.*, 2003).

The first step in a comparison of two or more sequences from evolutionarily-related genomes is to align the sequences in order to identify conserved regions. Two types of alignment programs exist, 'local' and 'global'. Local alignment tools produce optimal similarity scores between subregions of sequences, for example in cases where sequences exhibit conservation of gene synteny but jumbled order. These algorithms find short common segments between sequences first, and then extend the match as far as possible. Examples of local alignment tools are *BLASTZ* (Schwartz *et al.*, 2000) and *MUMmer2* (Delcher *et al.*, 2002); used to generate the alignment depicted in Figure 1). Global alignment tools produce optimal similarity scores over the entire length of the sequences being compared, for example in cases where the sequences are expected to share similarity over their full length. These methods attempt to find an all-inclusive map between sequences, but can be memory intensive and time consuming. Examples of global alignment tools are *AVID* (Bray *et al.*, 2003), *GLASS* (Batzoglou *et al.*, 2000) and *OWEN* (Ogurtsov *et al.*, 2002); used to generate the global alignment depicted in Figure 4). Several visualization software tools are available for the production of graphical views of alignments, using either

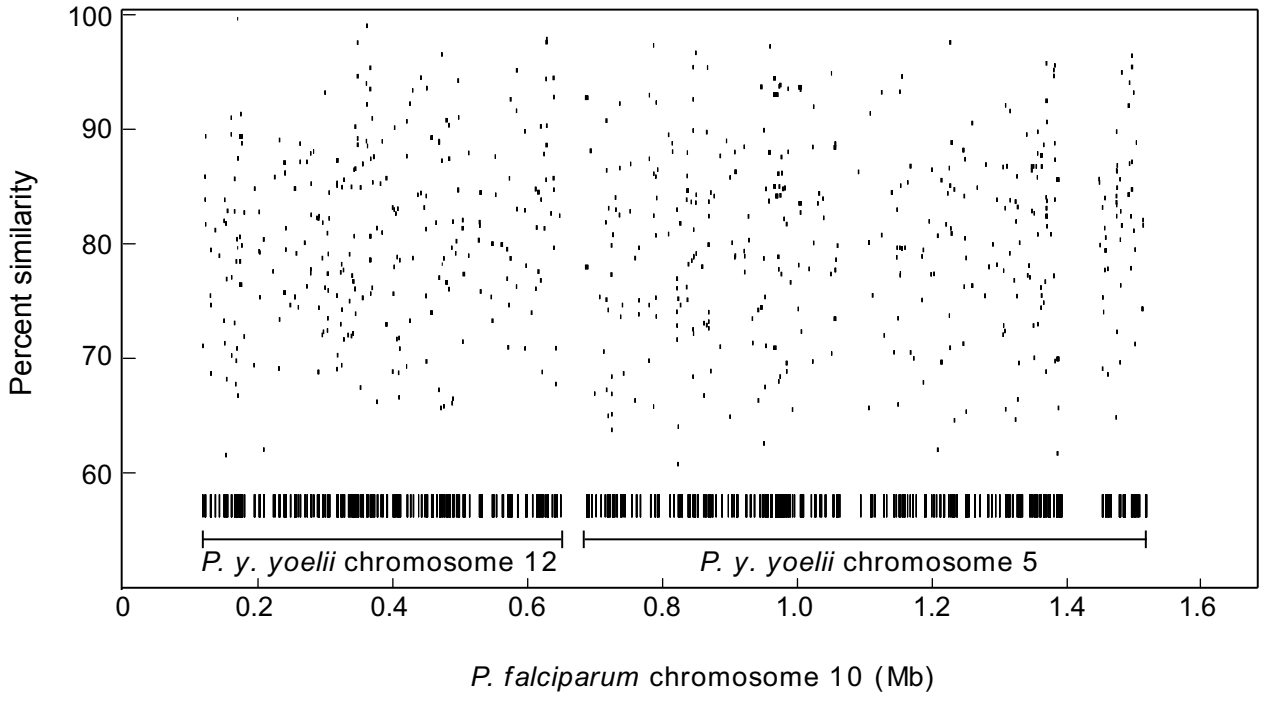

Figure 1. Schematic showing the tiling path of *P. y. yoelii* contigs along chromosome 10 of *P. falciparum* determined using *MUMmer*. The x-axis represents chromosome 10 (1.7 Mb), with vertical bars representing each *P. y. yoelii* contig that matches the *P. falciparum* chromosome. The percent similarity at the amino-acid level is given for each contig on the y-axis. The majority of contigs could be linked by PCR into two syntenic groups, and physical map data identified these as being located on *P. y. yoelii* chromosomes 12 and 5. Note the paucity of *P. y. yoelii* contigs with matches to the telomeric/subtelomeric ends of the *P. falciparum* chromosome, indicative of the species-specific immunodominant antigen genes located there.

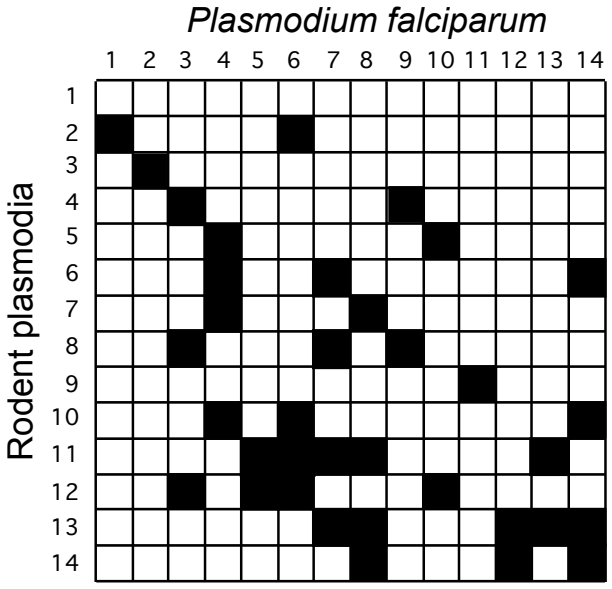

Figure 2. Genome-wide synteny map of *Plasmodium* plotted as an Oxford Grid. Syntenic regions conserved between *P. falciparum* and the rodent malaria species are shaded. For example, chromosome 8 in the rodent malaria parasites is syntenic to blocks of *P. falciparum* chromosomes 3, 7 and 9. The grid is incomplete as not all syntenic regions between the two species have been assigned to a rodent malaria chromosome. (Additional chromosome mapping data provided by T. Kooij and A. Waters, unpublished.)

local (eg *PipMaker* (Schwartz *et al*., 2000); *ACT* http://www.sanger.ac.uk/Software/ACT/, used to visualize the alignment in Figure 3) or global [eg *VISTA* (Mayor *et al*., 2000)] alignment software. Both local and global approaches to aligning sequences are informative; however, a comparison of alignment programs and servers is outside the scope of this essay. *MUMmer2*, *OWEN* and the alignment visualization tool *ACT* have all been used for comparative analysis of *Plasmodium* sequences by the authors and examples of these are given below.

Whole Genome Synteny Maps of *Plasmodium*

Using a mixture of computational algorithms and laboratory-based methods, a whole genome synteny map of the complete sequence of *P. falciparum* and the partial sequence of *P. y. yoelii* (Carlton *et al*., 2002) has been created. *MUMmer2* was used to identify local matches of at least five amino acids long from six-frame translations of both sequences; these seed matches were extended to create a tiling path of *P. y. yoelii* contigs against the *P. falciparum* chromosomes. The contigs were linked where possible by means of 'paired reads' and PCR amplification of the intervening sequence between contigs. The syntenic groups were assigned to a *P. y. yoelii* chromosome through the use of physical map data. An example of the tiling path of *P. y. yoelii* contigs against one *P. falciparum* chromosome is shown in Figure 1. From a total of 4,787 *P. y. yoelii* genes in the tiling path, 3,525 (74%) were found to be conserved in order between the two species using *Position Effect* (Carlton *et al*., 2002) software. This compares with 41/48 (85%) of genes found to be conserved in order in a 200 kb region syntenic between *P. falciparum* and *P. vivax* (Tchavtchitch *et al*., 2001).

The *P. y. yoelii*/*P. falciparum* syntenic map has identified long contiguous sections of the *P. y. yoelii* genome, and by extension, of the other rodent malaria parasite genomes, and their syntenic regions in *P. falciparum*. Studies are underway to complete and extend the map, which can be seen in its current form as an 'Oxford Grid' (a conventional method for displaying synteny between two species) in Figure 2. The construction of synteny maps between other *Plasmodium* species is also ongoing, although this is limited by the nature of the genome data. For example, creation of a map using partial genome data requires that one of the genomes be finished or at least in megabase 'scaffolds' and preferably with some karyotype and chromosome mapping data available. A synteny map of two human malaria species, *P. falciparum* and *P. vivax*, is planned, with preliminary tiling paths already suggesting a high degree of conservation of synteny between the two (Carlton, 2003). Synteny maps between *Plasmodium* species are particularly valuable for a number of studies: (1) as a means to chart the evolution of the genus, since syntenic break-points represent ancient evolutionary events that most likely occurred prior to speciation of the organisms being compared; (2) as a method of identifying true orthologs between species, for the comparison of molecular mechanisms underlying shared phenotypes; (3) for refinement of gene predictions through simultaneous annotation of multiple *Plasmodium* genomes; (4) for comparative analysis of gene expression, for example through identification of conserved non-coding regions of the *Plasmodium* genome ("phylogenetic footprinting"), and the evaluation of coordinated gene expression; and (5) as a means for the classification of genes under different evolutionary pressures. Examples of some of these studies are given below, many of which are works in progress due to the preliminary nature of *Plasmodium* comparative genomics.

Comparative Genomic Studies of *Plasmodium* Species

Molecular Evolution Studies of the Plasmodium Genus

Comparison of syntenic regions between *Plasmodium* species can aid in the creation of an evolutionary map of the genus. For example, DNA alterations leading to the generation of paralogous gene families, or to the loss or gain of genes in certain lineages, can be identified. Figure 3 shows an analysis of three genomes using *ACT*, a tool which reads annotated DNA sequences and *BLAST* analyses of the sequences and generates a visual map of syntenic regions. Two genes identified as coding for reticulocyte binding protein-2 (*RBP-2*) proteins are present in the *P. falciparum* genomic segment but absent in the *P. y. yoelii* and *P. knowlesi* contigs. These represent a gene family that appears to have been gained in *P. falciparum* or lost from the other species. In close proximity, the tandemly arrayed *MSP7*gene family appears to have undergone different degrees of gene expansion in the three species.

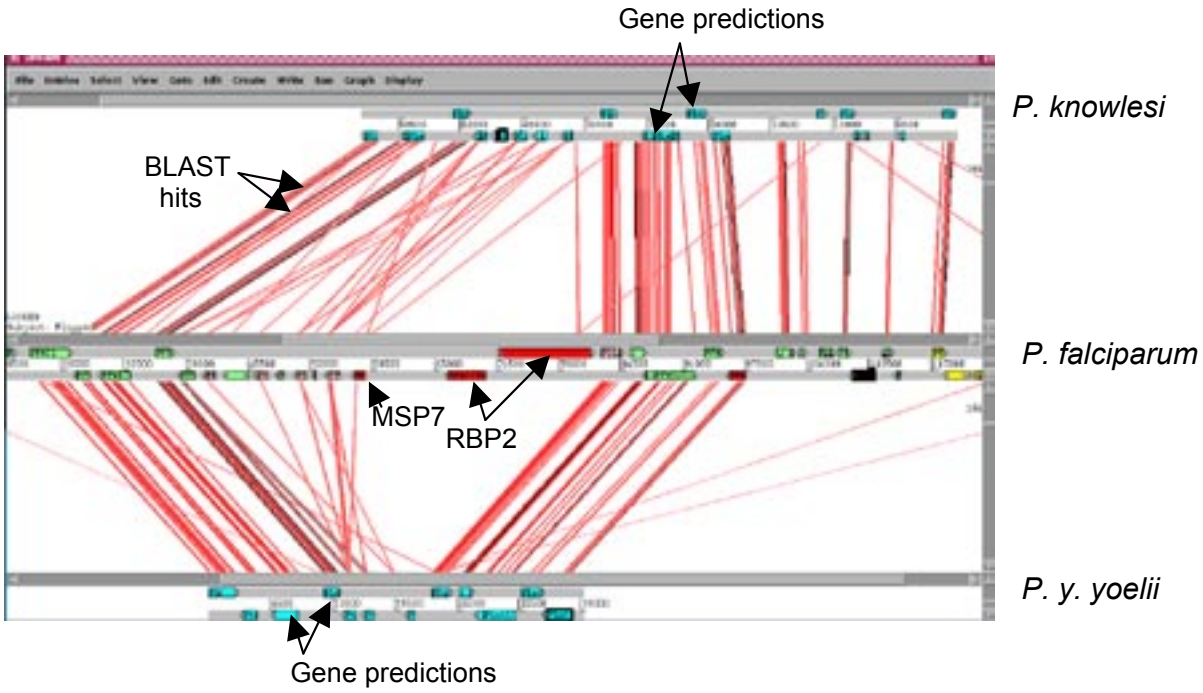

Figure 3. Graphical display generated by ACT of an alignment of a section of *P. falciparum* chromosome 13 compared to the syntenic regions in *P. y. yoelii* and *P. knowlesi*. Shaded, directional boxes indicate predicted genes on either DNA strand; *P. falciparum* gene predictions were manually curated. Vertical lines signify BLAST hits between the genomes. Two genes identified as coding for reticulocyte binding protein 2 (RBP2) proteins are present in the *P. falciparum* chromosome segment but absent in the *P. y. yoelii* and *P. falciparum* contigs. These represent a gene family that appears to have been inserted in *P. falciparum* or lost from the other species. The tandemly arranged *MSP7* gene family, located next to the *RBP2* genes, show various levels of gene expansion in the three species (present as three copies in *P. y. yoelii*, four copies in *P. knowlesi* and five copies in *P. falciparum*).

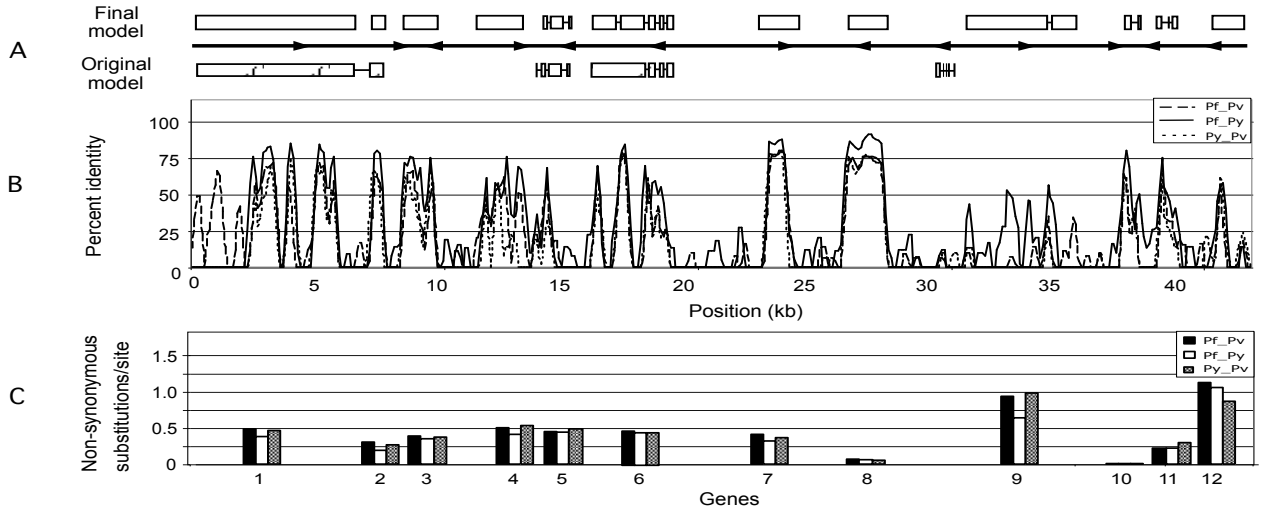

Figure 4. Global alignment of a 40 kb syntenic segment between three species of malaria parasite, *P. falciparum* (chromosome 3, at coordinates 178 kb to 220 kb), *P. vivax* (YAC1H14, at coordinates 95 kb to 135 kb) and *P. y. yoelii* (contigs MALPY2504, MALPY141, MALPY1025), encompassing twelve orthologous genes and 13 intergenic regions. (A) Structure of gene models used to estimate divergence are shown above the DNA strand (horizontal black line), and *P. falciparum* gene models refined through comparison with orthologous *P. vivax* and *P. y. yoelii* gene models are shown below the DNA strand. Gene orientation is represented by arrows. (B) Percent identity of the three pairwise nucleotide alignments, constructed using *OWEN* (Ogurtsov *et al.*, 2002) and computed using a sliding window of 250 bases with an overlap of 60%. Note the regions of conserved nucleotides in intergenic regions which may represent conserved non-coding regulatory regions. (C) Number of non-synonymous mutations per non-synonymous site plotted for all pairwise comparisons of the three species (see Carlton *et al.*, 2002 for methodology). Synonymous sites are saturated in all pairwise comparisons that include *P. vivax* (data not shown).

Generation of synteny maps between species can also help identify chromosomal rearrangement events that may have led to speciation. Several of the breaks in synteny between the *P. falciparum* and *P. y. yoelii* genomes were found to be located within areas containing the rRNA18S-5.8S-28S gene units, of which there are seven in *P. falciparum* (Gardner *et al.*, 2002), as well as in regions of the *P. falciparum* genome containing internal *var* and *rif* genes. Thus preliminary evidence exists for one possible mechanism underlying evolution of the *Plasmodium* genus, that of chromosome breakage and recombination at sites of rRNA genes (Carlton *et al.*, 2002).

Finally, the evolution of *P. falciparum* has been a matter of much debate (Hartl *et al.*, 2002), with one camp firmly of the view that *P. falciparum* is an ancient species, and the other that the species is of recent origin having emerged through one or several genetic bottlenecks. A genomics approach to tackling the question was undertaken recently with the creation of a SNP map of *P. falciparum* chromosome 3 from five parasite clones, which gave further credence to the view that the parasite is a genetically diverse and ancient species (Mu *et al.*, 2002). Comparative SNP studies of the syntenic region in *P. vivax* are underway and have provided evidence that *P. vivax* too has a highly diverse genome with an evolutionary history possibly parallel to that of *P. falciparum* (Feng *et al*., 2003**).**

Comparative Studies of Molecular Mechanisms Underlying Shared Phenotypes

Identification in one species of the ortholog of a candidate gene from a second species is important for cross-species comparison of gene function, and evaluation of molecular mechanisms associated with a shared phenotype. As an example, identification of the ortholog of the *P. falciparum* chloroquine resistance gene, *pfcrt*, in the *P. vivax* genome enabled comparison of the molecular mechanism of resistance to chloroquine in both species (Nomura *et al.*, 2001). A 350 kb YAC containing the *P. vivax* ortholog *pvcg10* was partially sequenced, and orthologs of genes in the same order and orientation as those flanking the *pfcrt* gene in the *P. falciparum* genome were identified, distinguishing *pvcg10* gene as the true ortholog of *pfcrt*. However, mutations in the *pvcg10* gene did not correlate with chloroquine resistance in *P. vivax* isolates, demonstrating that all pleiotropic functions are not necessarily shared between orthologs. Orthologs of the *cg10* gene from *P. knowlesi* and *P. berghei* were also sequenced and used to infer the ancestral haplotype of *pfcrt*. Since chloroquine-resistant *P. falciparum* isolates contain *pfcrt* alleles that deviate significantly from this haplotype, construction of the canonical sensitive allele through analysis of the gene in other model malaria species, enabled identification of the gene as being under strong selective pressure in *P. falciparum*.

Although in this instance the molecular mechanism underlying chloroquine resistance in two human malaria species was found to be different, rodent malaria models in particular have been used widely to study drug resistance in *P. falciparum* (Carlton *et al.*, 2001a). While the mechanism of resistance in some instances has been found to be remarkably similar between the species (such as the molecular basis for pyrimethamine resistance, which in many malaria species involves a single point mutation in the drug target dihyrofolate-reductase), the fact that the molecular mechanism can vary among different species does not negate the value of investigation into the phenotype in *Plasmodium* models. Such exploration provides an additional level of insight into the biology of the organism which may be valuable in other areas of *Plasmodium* research.

Gene Prediction and Annotation Refinement

Comparative genomics lends itself readily to the simultaneous annotation of syntenic regions in multiple species. Both gene models and accurate exon/intron boundaries can be difficult to predict in cases where little experimental evidence exists for verification, and where genome bias confounds the issue, as has been the case for gene prediction in *P. falciparum* (Gardner *et al.*, 2002; Hall *et al.*, 2002; Hyman *et al.*, 2002). Access to gene models from two or more species provides a way to check and improve on existing models, as shown in Figure 4. A global alignment of a 40 kb syntenic region from *P. falciparum*, *P. vivax* and *P. y. yoelii* shows that the structure and length of the gene models predicted in the three species using various gene prediction algorithms are in good agreement with each other. Four gene models in *P. falciparum* were altered to match those in *P. vivax* and *P. y. yoelii*; in all cases, the alternative *P. falciparum* model corresponded to an initial prediction made by one of the algorithms and subsequently discarded as a candidate for the final model. One gene model in *P. falciparum* (between genes 8 and 9) was excluded since it was not detected in either of the other species by any of the gene prediction algorithms. Thus, annotation of multiple *Plasmodium* genomes can aid in the verification and perfection of gene models in syntenic regions.

Phylogenetic Footprinting

Figure 4 also shows the power of global alignments for identification of conserved intergenic motifs (phylogenetic footprints) that may be involved in gene regulation. Little is known concerning DNA elements that direct the transciption of *Plasmodium* genes (Horrocks *et al.*, 1998; van Lin *et al.*, 2000). However, promoter elements from one species can function in other species (Crabb *et al.*, 1996), which indicates a significant functional conservation of elements between different *Plasmodium* species. As outlined above, alignment at the DNA level shows coding regions to be highly conserved between *Plasmodium* species, as shown by overlapping peaks and troughs of the pairwise comparisons that coincide with exons in the gene models in Figure 4B. (An exception in the example shown is gene 9, annotated as a hypothetical gene, for which very little similarity is found at the nucleotide level between *P. vivax* and the other two species. This difference is due at least in part to a marked shift in amino acid composition in this protein, with the (A+T)-rich codons coding for amino acids isoleucine, tyrosine, asparagine and lysine making up 50% of the protein in *P. falciparum* but only 20% in *P. vivax*, which exhibits a more balanced amino acid composition.) However, the pattern

of conservation within the coding regions differs markedly between genes; while some are conserved in their entirety (e.g., gene 10), others demonstrate fluctuation of conservation along the length of the gene (e.g., gene 1). In contrast, the similarity between species in intergenic regions is almost negligible, a situation mirrored in syntenic comparisons of mouse and human (Jareborg *et al.*, 1999). Since non-coding and silent positions in intergenic regions are mostly saturated (Carlton *et al.*, 2002), sequence similarity in these positions must be restricted to regions under selection. Prime phylogenetic footprint candidates are motifs conserved across all three species, some examples of which can be seen in Figure 4B. Phylogenetic footprinting has already been used successfully to detect conserved motifs in several eukaryotic lineages (Bergman *et al.*, 2001; Wasserman *et al.*, 2000; Webb *et al.*, 2002). Studies in *Plasmodium* will continue and expand to encompass alignment of genes known to be expressed at certain stages of the life-cycle (J. Silva and J. Carlton, unpublished).

Identification of Genes Under Selection Pressure

Multiple alignments of syntenic regions can be used in conjunction with simple molecular evolution methods to group *Plasmodium* genes according to the degree of selective pressure acting upon them. Similar methodology has been used on other organisms (Endo *et al.*, 1996), and in a few single gene studies in species of *Plasmodium* (Black *et al.*, 1999; Escalante *et al.*, 1998). With the release of large *Plasmodium* genome datasets, however, this can now be achieved on an automated whole-genome scale. As a detailed example, Figure 4C shows the number of non-synonymous substitutions (those that give rise to a change in amino acid) per non-synonymous site (d_N) for each of twelve orthologs in *P. vivax*, *P. falciparum* and *P. y. yoelii*. The degree of similarity in non-synonymous sites is roughly the same in the three pairwise comparisons for each gene, which suggests that these three species are approximately equidistant in evolutionary terms. However, the genes exhibit a wide spectrum of evolutionary rates, with some genes evolving under very strong 'stabilizing selection' (e.g., gene 10; $d_N = 0.01$) while others seem to be evolving under 'diversifying selection' (e.g., gene 12; $d_N > d_S > 1.0$). Differences in evolutionary rate among genes can be attributed to differences in the nature and degree of the selective constraints acting upon each gene. Comparison of d_N rates with gene function for genes 10 and 12 reveals that the highly conserved gene10 codes for the 60S ribosomal protein L44, a member of a highly conserved protein family found in widely divergent taxa such as mammals, protozoa and Archaea. In contrast, the highly divergent gene 12 codes for the circumsporozoite surface (CS) protein, a molecule found on the surface of *Plasmodium* sporozoites and known to interact directly with the host immune system. This class of gene is expected to differ greatly between species since its evolution is fast and dependent on interactions between each *Plasmodium* species and its host.

Since proteins of genes evolving under strong diversifying selection are likely to be in contact with the host immune system or to be targets of drug therapy, they represent good candidates for further study. Studies are underway to use this method to identify additional genes in this class (J. Silva and J. Carlton, unpublished). Furthermore, this analysis should identify species-specific genes that appear to be under diversifying selection in one species but not in others. In addition, extending this evolutionary analysis to encompass the whole genome will allow us to determine whether a non-synonymous divergence rate of 30% to 50% between the oldest branches of the malaria tree is indeed the norm.

The Future of *Plasmodium* Comparative Genomics

Comparative studies of model malaria parasites with the human malaria species they exemplify provide an invaluable additional level of insight into the biology of the organism and its interaction with host and vector. There is no doubt that model malaria species provide important knowledge through analogy or contrast with what is known concerning human malaria species. This interaction is set to be transfomed over the next few years as genome-wide comparisons of malaria species become possible on a scale not previously seen. Through the construction of genome-wide synteny maps, it will be possible to identify orthologs of human and model malaria parasites even in cases where sequence similarity is low in less well conserved genes, as is the case for many genes that encode surface-expressed proteins. Gene expression data from different transcriptome and proteome studies will enable the expression profile of a gene to be catalogued and compared in a variety of different species. However, further development of genetic manipulation technologies for use in *Plasmodium* will become increasingly necessary as a means to determine gene function and phenotype. High-throughput methods in particular, such as those developed for gene deletion-mutants in yeast (Giaever *et al.*, 2002) and RNAi in *Caenorhabditis elegans* (Kamath *et al.*, 2003), will be of immense value if they are transferable for use in *Plasmodium*.

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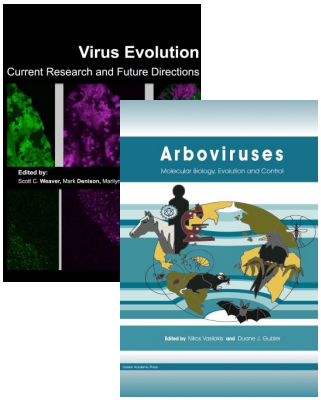

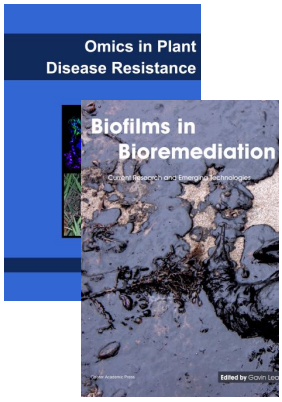

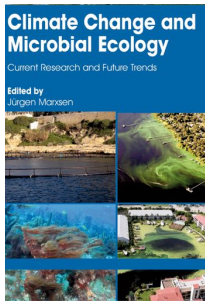

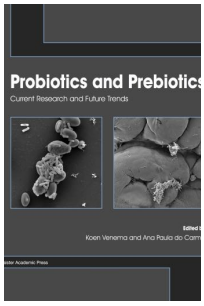

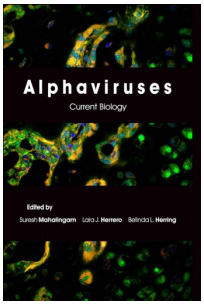

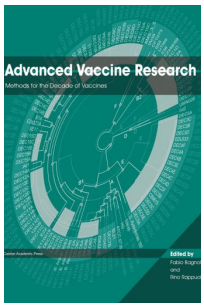

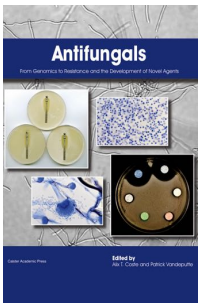

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