Current Biology, Vol. 14, 891–896, May 25, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.04.041

Genome Compaction and Stability in Microsporidian Intracellular Parasites

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Summary

Microsporidian genomes are extraordinary among eukaryotes for their extreme reduction: although they are similar in form to other eukaryotic genomes, they are typically smaller than many prokaryotic genomes [1, 2]. At the same time, their rates of sequence evolution are among the highest for eukaryotic organisms [3]. To explore the effects of compaction on nuclear genome evolution, we sequenced 685,000 bp of the *Antonospora locustae* genome (formerly *Nosema locustae*) and compared its organization with the recently completed genome of the human parasite *Encephalitozoon cuniculi* [1, 2]. Despite being very distantly related, the genomes of these two microsporidian species have retained an unexpected degree of synteny: 13% of genes are in the same context, and 30% of the genes were separated by a small number of short rearrangements. Microsporidian genomes are, therefore, paradoxically composed of rapidly evolving sequences harbored within a slowly evolving genome, although these two processes are sometimes considered to be coupled [4–7]. Microsporidian genomes show that eukaryotic genomes (like genes) do not evolve in a clock-like fashion, and genome stability may result from compaction in addition to a lack of recombination, as has been traditionally thought to occur in bacterial and organelle genomes [8–11].

Results and Discussion

Eukaryotic genomes are dynamic in their organization, such that gene order is typically conserved only among closely related species [12], but the forces that control genome plasticity are poorly understood [13]. Moreover, recent comparative genomic studies show that rates of genome structure evolution are variable among major animal lineages [5–7, 14, 15], although deeper, interkingdom comparisons are underexplored. Examples of gene order conservation at deep levels of divergence are known in a few cases where synteny is maintained either by chance [16] or because the proteins are part of a complex regulatory pathway [17]. In nuclear genomes of eukaryotes, rates of genome evolution appear to correlate with the overall rate of sequence evolution within a genome, probably because the occurrence and/or the probability of fixation of both nucleotide and chromosomal mutations are affected by the same genetic and life-history factor [4–6, 15]. Although this trend is also found in many prokaryotes, deviations from this correlation are also common in bacterial endosymbionts [9–11].

One eukaryotic group with exceptional genomic characteristics is the group of obligate intracellular parasites, microsporidia. Their genomes are among the most highly reduced in eukaryotes and their genes are among the most rapidly evolving at the sequence level [1–3], yet nothing is known about their genomic plasticity. Given a correlation between sequence evolution and genome plasticity, microsporidia would be predicted to have experienced a high rate of genome reshuffling during their evolution. To explore the dynamics of this process in these atypical eukaryotic genomes, we sequenced 729 fragments from a random genomic library of *A. locustae* (prepared according to [18, 19]), resulting in 685 Kbp of genomic sequence and 183 genes. Of these, 175 show significant similarity to *E. cuniculi* homologs, three show significant similarity to homologs in other organisms, but not *E. cuniculi*, and four are putative *A. locustae*-specific genes. As in *E. cuniculi*, the genome of *A. locustae* is highly compact, with gene density in the gene-rich regions of the *A. locustae* and *E. cuniculi* genomes being 0.94 and 0.97 genes per kilobase, respectively. Selecting fragments containing more than one partial or complete coding region yielded 44 fragments possessing two to six genes, for a total of 122 genes or 94 gene couples (see Table S1). The degree of conservation of gene order between *A. locustae* and *E. cuniculi* was measured as the percentage of gene couples in *A. locustae* that were also adjacent or close neighbors in *E. cuniculi*. Figures 1A and 1B show examples of conservation in order and direction over several genes. Figures 1C and 1D show two generally conserved regions with a few long-range and short-range rearrangements causing changes in order and orientation (see also Figure S1). In over 94 *A. locustae* gene couples, 13% were also adjacent in *E. cuniculi*, an additional 17% were close neighbors in *E. cuniculi*, and 43% of the *A. locustae* couples are located on the same chromosome in *E. cuniculi* (Figure 2). Recent data from yeasts [20], plants [21], nematodes [5], and mammals [22] show that a high proportion of small inversions are characteristic in eukaryotic genome evolution, whereas the opposite occurs in prokaryotes[23]. We examined the relative orientation of conserved couples in *E. cuniculi* and *A. locustae* and found that 12 of 27 adjacent or neighbor couples were inverted. Of these, eight arrangements could be explained by one inversion and four by two inversions, some involving one or a few genes. This proportion of small inversions is consistent with the behavior of other eukaryotic genomes and might reinforce the expectation that the gene order of microsporidia would be highly scrambled: while long-range rearrangements alter gross genome structure, small inversions tend to quickly disrupt short-range gene orders [21]. In contrast, however, *E. cuniculi* and *A. locustae*

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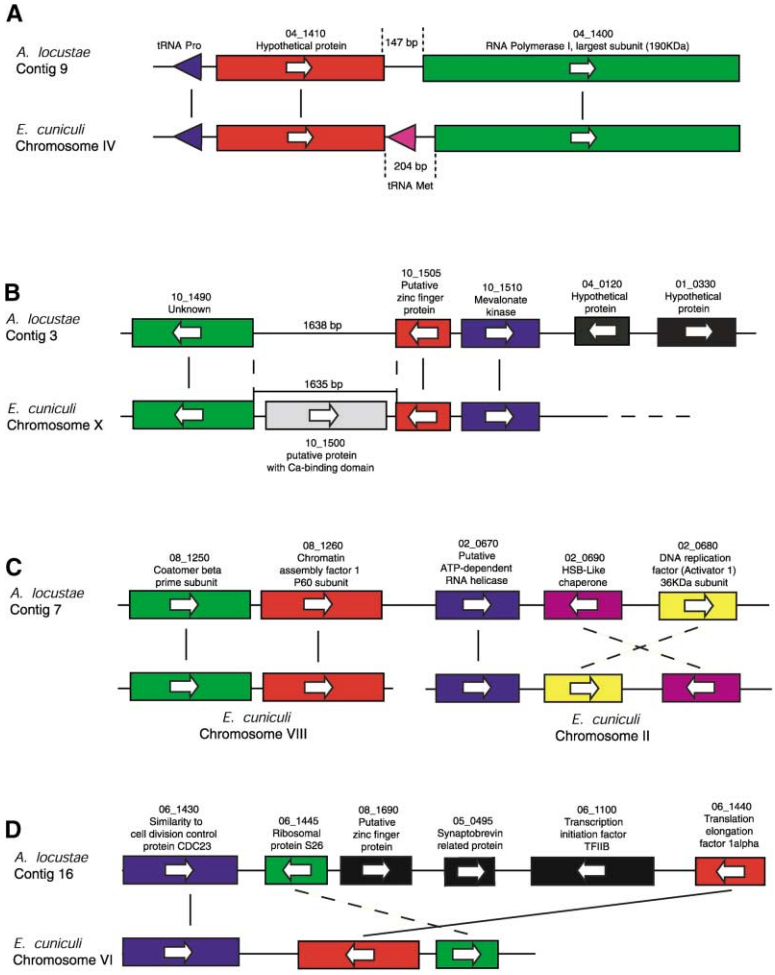

Figure 1. Examples of Gene Order Conservation and Divergence between *A. locustae* and *E. cuniculi*

Genes were identified by similarity through BLAST searches against NCBI databases using Netblast release 2.2.4 and the web-based BLAST interface (http://www.ncbi.nlm.nih.gov/blast) with the programs blastx, tblastn, and blastp. Internal searches were performed with a modified version of ESTid (generously supplied by M. Reith). Positional information on *E. cuniculi* genes was retrieved from The Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg). In each scheme, the upper part corresponds to an *A. locustae* genomic fragment and the lower to an *E. cuniculi* fragment. Genes are labeled according to their designation in the *E. cuniculi* genome (e.g., 04_1200 denotes chromosome IV, locus 120), and transcriptional direction is indicated by arrows. Conserved genes are color coded and joined by a solid line if the orientation is conserved and a broken line if they have been inverted. Open reading frames and intergenic regions not shown in scale. (A and B) Examples of conservation in order and orientation. (C and D) Conservation of gene order with one (C) or more (D) inversions.

share a seemingly high proportion of adjacent genes (13%) and genes separated by no more than five other genes (26%) (Figure 2).

The significance of these figures can only be interpreted in light of the degree of relatedness between the species. A molecular phylogeny using the SSU rRNA sequence shows that *A. locustae* and *E. cuniculi* are very distantly related within microsporidia (Figure 3A). Absence of fossils and the rapid rate of sequence evolution in microsporidian genes prevent any direct comparison between the rate of genome shuffling in microsporidia with that of another eukaryotic group. However, a relative comparison to the ascomycete fungi is still informative, since fungi are the closest relatives of microsporidia [1, 24, 25], and complete or substantial genome data exist for several ascomycetes. Figure 3B shows a fungal phylogeny indicating that *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Candida albicans* span the diversity of ascomycetes but are comparatively closely related in the broader view of fungal diversity. The level of gene order conservation between the microsporidia (13%) is almost 1.5-fold higher than that between *S. cerevisiae* and *C. albicans* (9%), while there is no detectable conservation between

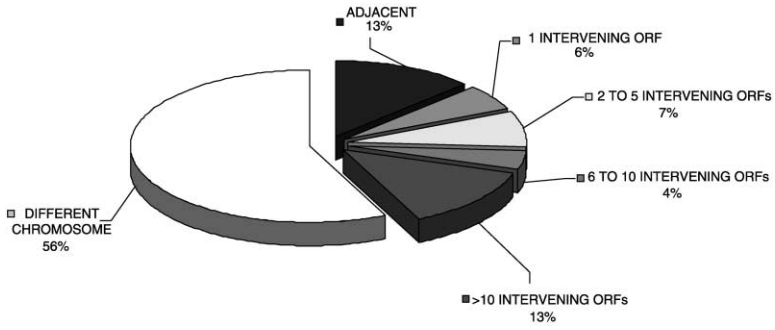

Figure 2. Conservation of Gene Oder between *A. locustae* and *E. cuniculi*

Each category displays the percentage of gene couples that are adjacent in *A. locustae* and adjacent, close neighbors or nonsyntenic in *E. cuniculi*. All pairs of adjacent genes in the *A. locustae* sequences were counted as couples (putative ORFs not present in *E. cuniculi* were ignored). Genes that are adjacent in *E. cuniculi* and are separated by a few intervening genes in *A. locustae* are also included.

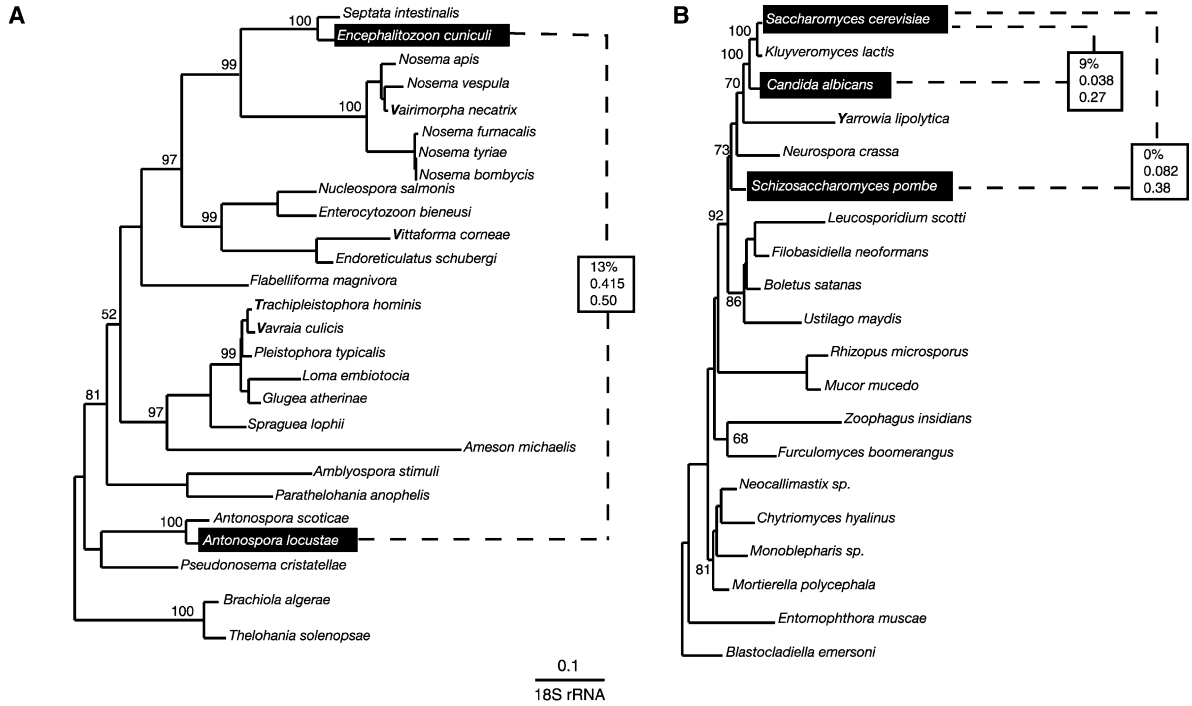

Figure 3. Comparison of Evolutionary Relatedness between Microsporidia and Fungi

Molecular phylogenies of microsporidia (A) and fungi (B) based on SSU rRNA (see Table S4 for accession numbers) aligned with ClustalX. Gaps and poorly aligned regions were excluded. Maximum likelihood trees were inferred with PAUP* 4.0b10 [42] by using GTR, γ, and invariable sites. Numbers at nodes correspond to percentage of bootstrap support over 500 pseudoreplicates. Data for *Candida albicans* was obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida. Boxes linking pairs of taxa indicate percentage of adjacency conservation, SSU rRNA distance, and average protein p distance among 14 proteins shared by all five species (see Table S2 for details). Scale bar represents the number of (corrected) changes per site, and both trees are drawn to the same scale.

S. cerevisiae and *S. pombe* [20, 26]. In contrast, the levels of sequence conservation between microsporidian SSU rRNA are 10- and 5-fold lower than that between *C. albicans* and *S. cerevisiae*, and *S. cerevisiae* and *S. pombe*, respectively. Likewise, average divergence at the protein sequence level was substantially higher for the microsporidian species than for the *S. cerevisiae*-*C. albicans* and *S. cerevisiae*-*S. pombe* pairs, respectively (Figure 3 and Table S2).

To extend this comparison to animal genomes, we have also examined gene order conservation between humans and teleosts. Using data from the nearly complete (95% coverage) *Fugu* genome [27], we estimate that 4% of *Fugu* genes have human homologs that are strictly adjacent, in line with previous analyses [28], which showed that 11% and 18% of *Fugu* genes pairs are separated by no more than five and ten intervening genes in humans, respectively. As with ascomycetes, the level of sequence evolution between humans and teleosts is significantly lower than that between microsporidia (See Table S2). Levels of synteny between other animal genomes, mainly involving nematodes, dipterans and mammals [5, 6, 15, 29], have been estimated by using marker-based methods[30]. However, these cannot be directly compared with microsporidia, since synteny is determined by the length of conserved segments instead of proportion of adjacent or neighbor genes, which lacks resolution at the level of single genes. Notwithstanding, these studies point to nematodes and dipterans as the animal lineages with the fastest rates of genome rearrangements [5–7], so it is unlikely that the human-fish estimate of synteny is particularly low. Among plants, the genomes of grasses were considered to be highly conserved in structure, but recent sequence analyses have shown that thousands of rearrangements have differentiated grass genomes since their relatively recent origin ($\sim$50 mya [million years ago]) [21, 31]. Moreover, comparisons between grasses and *Arabidopsis* show that few colinear segments are shared between monocots and dicots [32]. Although less conclusive, data from plant genomes suggest that only residual colinearity would remain at deep levels of divergence. Information from eukaryotes other than animals, plants, and fungi is severely limited, but whole genome data are available for some apicomplexan parasites. Human and rodent malaria agents *P. falciparum* and *P. yoelii* diverged comparatively recently and share many segments of conserved gene order, but their synteny has not been quantified in terms of gene adjacency [33]. *Cryptosporidium parvum* and *P. falciparum* represent a much more ancient divergence and show no significant conservation of gene order [34]. Unfortunately, however, the significance of these comparisons is still unclear because their evolutionary history is still poorly understood, as are the dates of their divergence.

If we reconsider the comparison between microsporidian and ascomycete genomes, there are two opposing explanations, and both have significant implications. On one hand, if we consider genomic shuffling in microsporidian and ascomycete genomes to be taking place at similar rates, then microsporidia would have to have originated sometime after the divergence of the budding yeasts, *C. albicans*, and *S. cerevisiae* (Saccharomycetales), which are estimated to have diverged only about 200 mya [35]. Microsporidia almost exclusively infect animals, where they have been described from all phyla, and are found to infect a broad range of species within each phylum in virtually all ecological contexts. For microsporidia to have originated less than 200 mya (between the origin of mammals and the placental-marsupial split [36]), they must have invaded a single animal host and then quickly spread throughout the kingdom in an explosive radiation, affecting distantly related hosts in a wide variety of ecological niches. This would not only be an astonishing "infection" of a kingdom, but also would be inconsistent with the nature of modern microsporidia, since most infect a single host or a comparatively narrow host range [37, 38]. Phylogenetic analyses have also showed than related microsporidia tend to infect similar hosts and have inferred only a handful of "jumps" between distantly related animals (e.g., [39]).

The alternative explanation is that the structure of microsporidian genomes evolves slowly compared to fungal genomes, a suggestion that is consistent with the extreme compaction of microsporidian genomes. The gene density in the gene-rich regions of the *A. locustae* and *E. cuniculi* genomes are both about one gene per kilobase, while the density of other eukaryotic genomes is typically much lower: *S. cerevisiae*, which is often considered compact, has a gene density of half that seen in microsporidia. Inversions and transpositions generate breakpoints in the genome, and the greater the likelihood that these breakpoints will fall within a functionally important gene or regulatory region, the lower the likelihood that the rearrangement will succeed. Highly reduced prokaryotic genomes have shown similar stasis [10], the best example of which can be seen in mitochondria. Vertebrate mitochondrial genomes are extremely compact and highly conserved in their overall structure. In contrast, angiosperm mitochondrial genomes are much larger, the genes distantly spaced, and there is virtually no conservation in gene order. In addition, plant nuclear genomes maintain little colinearity across the mono and dicotyledonous boundary, but conserved clusters of genes can be observed in small regions of densely packed genes with short intergenic segments [31]. In both bacteria and mitochondria, reduced genomic plasticity has been partially attributed to the reduction or complete absence of recombination and the absence of key proteins such as RecA [8–11]. However, microsporidia are sexual, and their recombination systems do not appear to be attenuated, suggesting recombination alone will not explain their genomic stability [1, 3].

The importance of compaction on genomic stability is also supported by a specific prediction regarding the distribution of lengths of intergenic regions. If the conservation in gene order is affected by compact intergenic regions, then the intergenic regions between conserved gene pairs should be shorter on average than the intergenic regions between nonconserved pairs. Indeed, the average distance between gene pairs that are adjacent is less than half the distance between genes that are not adjacent in both *A. locustae* and *E. cuniculi* (Figure 4). This correlation would not be expected if microsporidian genome flexibility were not constrained by compaction. Adaptive explanations such as coregulation of functionally associated genes have also been postulated to explain conserved gene order [23, 40]; however, we found no evidence of clustering of functionally related genes. Pairs of functionally associated genes have previously been found to be linked in microsporidian genomes [1, 2, 18], but such clustering will appear more often in highly reduced genomes simply by chance. As a genome is reduced, gene loss is not random. Instead, much reduction occurs in packages representing whole pathways, so the remaining repertoire of genes is distributed among a smaller number of essential functional categories. Accordingly, the likelihood of any gene falling beside another of functional relationship is much higher, even when genes are distributed randomly. Indeed, calculating the distribution of functionally related gene pairs shows that the observed numbers of same-category couples is consistently lower than what is expected by random arrangement (Table S3). It is possible that genome stasis is, in part, result of loss or severe reduction in the number of mobile and repeated elements as observed in *E. cuniculi* [1], since it is known that repeated sequences are an important vehicle for rearrangements [14]. However, whether this is generally true of microsporidian genomes remains to be seen, since evidence of a transposable element has been reported in *Vittaforma corneae* [41], and repeated sequences exist in *A. locustae* [19].

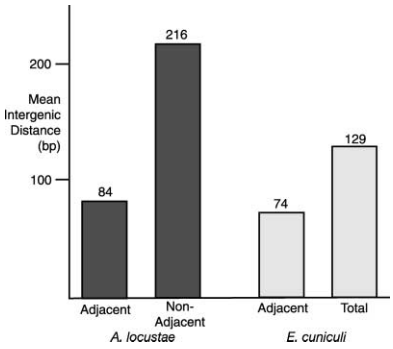

Figure 4. Relationship between Intergenic Distances and Gene Adjacency

Mean intergenic distance (in bp) between genes. Dark gray, *A. locustae* intergenic distances between genes that are adjacent or nonadjacent in *E. cuniculi*. Light gray, *E. cuniculi* intergenic distances between genes adjacent in *A. locustae* and mean intergenic distance of all *E. cuniculi* genes [1].

Microsporidia provide the first comparative look at the evolution of a highly specialized and compacted eukaryotic genome. The rate of sequence evolution in microsporidian genomes is strikingly high, while the rate of genomic reorganization is low. One of the most important advances in molecular evolution has been the rec-

ognition that sequences do not follow a clock-like behavior. Microsporidian genomes show that this can be extended to the evolution of the eukaryotic genome itself.

Supplemental Data

Supplemental Data including additional schemes and details on sequences used in this paper and four Supplemental Tables are available at http://www.current-biology.com/cgi/content/full/14/10/891/DC1/.

Acknowledgments

We thank M. Reith for the use of ESTid, and J.M. Archibald and B.A.P. Williams for discussion and comments. This work was supported by a New Investigator Award to P.J.K. from the Burroughs-Wellcome Fund. N.M.F. is supported by fellowships from Michael Smith Foundation for Health Research (M.S.F.H.R.) and the Canadian Institutes of Health Research (C.I.H.R.). P.J.K. is a New Investigator of the MSFHR and CIHR and Scholar of the Canadian Institute for Advanced Research. Sequencing of *Candida albicans* was accomplished with the support of the National Institute of Dental Research and the Burroughs Wellcome Fund.

Received: November 24, 2003
Revised: March 22, 2004
Accepted: March 22, 2004
Published: May 25, 2004

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Accession Numbers

Accession numbers AY548884–AY548926 correspond to each of the DNA sequences listed in Table S1. Each sequence has more than one gene product, all of which are listed in the "open reading frame description" column in Table S1.

Accession numbers AY574349–AY574351 correspond to three additional (also new) DNA sequences, each with a single gene product, all of which are indicated in Table S4: Elongation factor 2, Deoxyuridine 5′ triphosphate nucleotidehydrolase, and Ribosomal protein L1.