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Oxytricha as a modern analog of ancient genome evolution

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Abstract

Several independent lines of evidence suggest that the modern genetic system was preceded by the 'RNA world' in which RNA genes encoded RNA catalysts. Current gaps in our conceptual framework of early genetic systems make it difficult to imagine how a stable RNA genome may have functioned and how the transition to a DNA genome could have taken place. Here we use the single-celled ciliate, *Oxytricha*, as an analog to some of the genetic and genomic traits that may have been present in organisms before and during the establishment of a DNA genome. *Oxytricha* and its close relatives have a unique genome architecture involving two differentiated nuclei, one of which encodes the genome on small, linear nanochromosomes. While its unique genomic characteristics are relatively modern, some physiological processes related to the genomes and nuclei of *Oxytricha* may exemplify primitive states of the developing genetic system.

Early genome evolution

The modern genetic system requires the synthesis and functional orchestration of three distinct biopolymers: DNA, RNA, and proteins. This complex system was likely preceded by a stage in which RNA played a central role both in information storage and as the only genetically-encoded catalyst (Figure 1) [1,2]. The early prominence of RNA is substantiated by its ability to store genetic information, as in mRNA, and to impart catalysis, as demonstrated by the abundance of catalytic RNAs present in nature and produced in laboratories [3]. The primacy of functional RNAs in the process of protein translation (transfer and ribosomal RNAs and other functional RNAs that modify them), coupled to the ubiquity of those RNAs across all extant life, suggests that the translation system emerged from an RNA-catalyzed metabolism [4]. The central role of nucleotide-derived cofactors (such as ATP, NADH, and CoA) in metabolism is consistent with a scenario in which those functions were previously catalyzed by ribozymes [5].

The catalytic range of RNA is limited and a ribozyme-based metabolic system probably remained dependent on the background chemistry from which it emerged. The development of protein translation may have evolved as a mechanism to bring this crucial chemistry under the control of genetically-encoded enzymes [6]. Deoxyribonucleotides were probably unavailable until the evolution of ribonucleotide reductase proteins [7], implying that the development of the DNA genome was not even possible until substantial evolution of protein enzymes had taken place. By this point, the translation system seems to have reached a moderate level of its modern sophistication and the range of protein fold architectures encoded by early genomes had significantly expanded [8].

The transition from an RNA genome to a DNA genome is not well understood. Many protein fold architectures seem to have evolved before this stage, because modern

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ribonucleotide reductase enzymes fall into three distinct classes that share no noticeable similarity in amino acid sequence but appear to be homologous when their active site amino acids are compared in 3D structure alignments [9]. Although DNA-processing functions are similar across the tree of life, no ancient core of enzymes can be detected by sequence comparison (Figure 2) [10]. Six distinct families of DNA polymerase are known, but only those with specific functions related to excision repair have a universal taxonomic distribution [11]. Two distinct families of DNA primase, one bacterial and one archaeal/eukaryotic, are observed in modern life. A similar phylogenetic pattern is observed in DNA ligases. This lack of a universal DNA metabolism may imply that a complete protein-catalyzed DNA-processing system was not present in the last universal common ancestor (LUCA) or that ancient non-orthologous gene displacements [12], in either the ancestor of Bacteria or the ancestor of Archaea and Eukarya, erased the phylogenetic evidence of most DNA processes in LUCA.

In lieu of the current inability to reconstruct early genome-related metabolism through bioinformatics, some researchers have used features of modern biological systems as analogs to traits of ancient organisms and their genomes. For example, it has been argued that viruses provide an ideal evolutionary platform to acquire a DNA genome in an RNA world and to distribute this trait to cellular life [13]. A similar approach compares the notion of early genomes to a ciliate macronucleus in which genes are encoded on small linear chromosomes [14]. Here, we expand the latter idea and use the remarkable genetic system of the ciliate genus *Oxytricha* [15] to improve our understanding of the early transition from RNA to DNA genomes.

Oxytricha

Oxytricha is a genus of single-celled ciliated protists. They are predatory, mitochondrion-bearing, free-living organisms that inhabit freshwater environments. Its lineage diverged ~1 Gya ago from the common ancestor of *Tetrahymena* and *Paramecium* [16]. *Oxytricha* spp, like most ciliates, have two types of nuclei, a micronucleus and a macronucleus (reviewed in [17]). The macronucleus is transcriptionally active during vegetative growth, whereas the micronucleus is almost always transcriptionally silent. However, only the micronucleus is exchanged during the ciliate sexual cycle, after which a new macronucleus and macronuclear genome are formed from micronuclear DNA. Although these general traits are common throughout the phylum Ciliophora, the architectures of the macronuclear and micronuclear genomes, as well as the process of macronuclear development, differ among ciliate taxa.

The *Oxytricha* micronuclear genome contains approximately 1Gb of sequence, while the macronuclear genome contains approximately 50Mb of sequence, representing a 95% reduction in genome content during development [16]. In addition, thousands of micronuclear genes are scrambled with respect to their macronuclear counterparts, with segments of micronuclear genes present in a permuted or inverted order relative to their order in the macronucleus (Figure 3) (reviewed in [18]). Following sexual exchange of haploid micronuclei, the macronuclear genome assembles from dispersed segments of micronuclear DNA through a process of genome rearrangement that is guided by macronuclear RNA templates (Figure 3) [19]. It is likely that these RNA templates represent a transient cache of the entire macronuclear genome during this developmental stage.

The roles of RNA may surpass those of DNA in regulating the information in the genome of *Oxytricha* at three levels. At the first level, RNA transcripts of complete nanochromosomes from the previous generation can program the pattern of DNA rearrangements during macronuclear development [19]. The microinjection of synthetic RNA molecules into

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Oxytricha cells can introduce an alternative order of micronuclear DNA segments in the resulting progeny [18,19]. These new DNA rearrangement patterns can transfer to the sexual offspring of those progeny and even their progeny's progeny. Given that the micronuclear DNA remains unchanged, the inheritance of altered rearrangement patterns in *Oxytricha* appears to be a transgenerational RNA-mediated epigenetic phenomenon.

At the second level, point substitutions can also transfer from the RNA template to the macronuclear DNA [19], particularly near regions where junctions form between macronuclear segments. These point substitutions can also transfer to the sexual progeny and their progeny's progeny. Given that the micronuclear DNA does not share these point substitutions [19], this observation implicates a role for RNA-templated DNA repair [20] in DNA rearrangement. These somatically acquired point mutations represent another level at which epigenetically-inherited RNA molecules instruct the sequence and interpretation of the DNA genome.

At the third level, the RNA macronuclear genome cache also appears to be responsible for determining the copy number of macronuclear chromosomes. Artificially increasing or decreasing the available levels of RNA chromosome templates by microinjection or RNAi, respectively, leads to a relative increase or decrease in the copy number of the corresponding DNA molecules in the next generation. This effect also lasts at least two sexual generations [21], demonstrating a further example of RNA-mediated transgenerational epigenetic inheritance in *Oxytricha*.

Apart from its unique sequence features, ciliate micronuclear genomes have a normal eukaryotic structure. Their genome architecture is in the form of large chromosomes with telomeres and a centromere, and micronuclei reproduce via mitosis during cell division. During the sexual cycle the diploid genome undergoes meiosis to produce haploid gametes, one of which is retained and the other of which passes to the mating partner (Figure 3) [22,23].

The macronucleus is very different. The macronuclear genome contains on the order of 20 million small DNA chromosomes, or 'nanochromosomes', most of which encode a single protein-coding gene or functional RNA. In fact, the lack of a centromere has led some to argue that the term 'chromosome' is inappropriate for macronuclear DNA [16]. The extraordinary number of DNA molecules in the macronucleus results from approximately 20 000 unique nanochromosomes averaging roughly 1000 copies per macronucleus. Their average length is approximately 2.7 kb [24]. These unusual properties of the *Oxytricha* macronuclear genome and macronucleus, and the powerful role of RNA in sculpting these genomes, offer a compelling system within which to consider possible transitions from simple RNA genomes to complex DNA genomes.

Oxytricha and early genome replication

Small, single-gene chromosomes, such as those in the *Oxytricha* macronucleus, represent one of the simplest possible states of a genome and thus were probably predecessors to more complex genome architectures. A genome of small linear chromosomes would have presented less of a challenge to primitive polymerases [14], which probably copied nucleic acids with low fidelity and were unable to process long sequences. The nature of these primitive DNA polymerases is unknown. None of the four families of standard DNA polymerases has a universal distribution [11], although the sliding clamp function of the DnaN polymerase in *E. coli* and the 5′–3′ exonuclease function of the *Pol1-A* polymerase in *E. coli* appear to have been present in LUCA [25,26]. Three subunits of DNA-dependent RNA polymerases appear to be universal as well [25]. Structural and functional comparisons

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of DNA-dependent RNA polymerases suggest that they may share a multi-subunit ancestor with proofreading capabilities that was present in LUCA [27].

It is generally assumed that the RNA-only stage in the development of the genetic system would have required an RNA-dependent RNA polymerase ribozyme to have replicated the genome. Although no such enzyme has been found in extant biology, several have been produced synthetically through laboratory evolution techniques [24,28-30]. So far, all of these ribozymes are over a hundred nucleotides long and exhibit very tight constraints on sequence space, making it difficult to imagine how similar ribozymes could have evolved *de novo* in an RNA world scenario. In addition, even the most capable of these laboratory-generated polymerase ribozymes is not able to sustain the processivity required to replicate RNA molecules of its own size or larger.

During the process of *Oxytricha* genome rearrangement, segments of DNA from the micronuclear genome assemble according to RNA templates of the macronuclear genome (Figure 3). This process represents a unique scenario in extant biology in which a complete copy of a genome is produced, not by polymerizing a complementary strand one nucleotide at a time, but by recycling DNA polymers from a precursor genome. It is likely that these pieces of micronuclear DNA ligate together after assembling on the complementary RNA template, although there is also evidence that gaps or errors between the DNA segments are repaired by the activity of an RNA-dependent DNA polymerase [19].

A similar mode of replication would have conferred several benefits to early life and perhaps created a viable selection regime in which polymerases with high fidelity and processivity might have evolved. In contrast to ribozyme polymerases, several ribozyme ligases are present in modern organisms [3] and more have been synthesized by directed evolution [31,32]. Polymerases are in fact a specialized kind of ligase in which one of the ligated partners is a single nucleotide [31]. It follows, then, that the central challenge to a polymerase is not the catalytic step of ligation, but the ability to perform that step repeatedly over the full length of a gene-sized molecule, a limitation that is borne out by the difficulty of producing a highly processive ribozyme polymerase [24,29,30].

If early nanochromosomes replicated in an *Oxytricha*-like fashion, the number of catalytic ligation steps would be much smaller than that in a complete polymerase-dependent replication. The source of these DNA segments in a primitive system is not clear. Perhaps if the GC% was very high or very low, the sequence complexity of the nanochromosomes would also be low, and short abiotically synthesized segments with random sequences [33,34] would provide enough matches to the template to permit assembly of most of the genome from these small, modular pieces [35]. The need to fill or repair small gaps between segments would create a selective environment for the evolution of a weakly processive polymerase into the ancestor of a modern, highly processive polymerase. This model of early genome replication is consistent with the observation that the only universally conserved DNA polymerase families are involved in excision repair (Figure 2). Once a high-fidelity, high-processivity polymerase became available, genome replication could move towards its current polymerase-dependent form and longer chromosome lengths would be possible.

Oxytricha and early cell division

In most Eukaryotes, cell division is orchestrated by the complex process of mitosis, wherein duplicate chromosomes segregate evenly between the dividing cells. The process is controlled by dynamic motor complexes that pull chromosomes along organized microtubules [36,37]. Functionally analogous but non-homologous processes are thought to take place in Bacteria [38-40] and Archaea [41,42]. It is difficult to imagine that such a

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complex system was present in early life forms. Early cell division probably involved uncontrolled membrane division with chromosomes segregating at random.

Similarly, the *Oxytricha* macronucleus does not divide by way of mitosis. The approximately 20 million nanochromosome molecules probably present an overwhelming challenge to organized mitotic segregation. Although amitosis in *Oxytricha* is microtubule-dependent [43,44], these microtubules appear to control membrane division rather than chromosome segregation. Macronuclear nanochromosomes lack centromeres to which mitotic motors, or kinetochores, would normally attach [16,45]. As a result, the segregation of DNA between macronuclei is unpredictable and often uneven [16,46]. Amitotic division of macronuclei seems to have arisen early in the ciliates [47] although previous phylogenies have predicted three independent origins of amitosis in ciliates, with one origin in the common lineage of the genera, *Oxytricha* and *Euplotes* [48].

It is possible that the high chromosome copy-numbers observed in *Oxytricha*, and to an equal or lesser extent in other ciliates, are related to the imprecise segregation of chromosomes during amitotic division [49]. If a single chromosome is duplicated and the two copies are allowed to segregate randomly to one of the two daughter cells, then the probability of losing that gene in one of the daughter cells is 0.5. A greater number of chromosomes will statistically ensure an approximately even segregation of the chromosomes between daughter cells. This feature of amitosis in *Oxytricha* may be similar to the division of primitive cells, which would have also benefited from carrying chromosomes in high copy-numbers to safeguard against uneven segregation.

Oxytricha and early genome stability

A single common ancestor of all life is the most statistically satisfying explanation for common traits observed in modern organisms [50]. This explanation, however, does not distinguish between a single organism and a community of organisms with highly pervasive lateral gene transfer [14]. Even if we assume the former scenario, the complexity of a single LUCA organism may have been generated in part by lateral gene transfer within a heterogeneous population of organisms [26]. If early genomes did indeed resemble *Oxytricha* macronuclear genomes, then the nature of the RNA-mediated gene transfer observed in *Oxytricha* [19,23] may also help describe the sort of communal inheritance that preceded the predominantly vertical inheritance of modern organisms.

The nanochromosome structure of the macronuclear genome and its regeneration through RNA-template-directed DNA unscrambling provide a form of lateral gene transfer that differs from mechanisms described in any other organisms. Unlike conventional conjugation in bacteria or sexual reproduction in eukaryotes, an RNA-driven epigenetic mode of inheritance does not require the introduction of new genes, but instead new ‘alleles’ can spread via conversion of existing ones (through RNA-guided mechanisms). Allele frequencies can be increased or decreased by the introduction of foreign nucleic acids, and these acquired traits are passed on to subsequent generations.

These phenomena are similar to horizontal gene transfer, in that somatic DNA or RNA variants provide an external source of genetic variation. But the nanochromosome structure of the macronuclear genome and its capacity to receive new alleles during the process of DNA arrangement make the *Oxytricha* macronucleus uniquely permissive to somatically acquired genetic change. Nevertheless, an epigenetic system such as that of *Oxytricha* is also robust to such perturbations because the high copy-number of original alleles will initially act as a buffer against sequence change, restricting the spread of deleterious somatic alterations. Perhaps early genomes with structures similar to the *Oxytricha* macronucleus would also be permissive to genetic acquisitions, but stable against their deleterious effects.

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Oxytricha and early organismal identity

The genetic openness that existed during the transition to modern life was probably also prone to invasions by selfish replicators that may have easily infiltrated and taken advantage of emerging organismal replicating systems [51]. This effect is generally modeled through self-propagating metabolism-like networks, or hypercycles. These replicating entities may be parasitic if they either receive replication support from the host system without conferring a reciprocal benefit, or shortcut the host system in some deleterious way. Vesicles can barricade replicating systems against selfish entities if they provide a mechanism of blocking the entry of external replicators [52]. Selfish replicators can also be eventually incorporated into the metabolism of the host system, balancing their deleterious effects with beneficial ones [53].

Although the dynamics of nuclear dimorphism in *Oxytricha* do not resemble a hypercycle, the scrambling of the micronucleus and its rearrangement to form the macronuclear genome illustrate the properties of stable systems that host selfish replicators. The unique genomic traits of *Oxytricha* seem to be both caused by and assisted by an invasion of DNA transposons (typically regarded as selfish genetic agents). The micronucleus hosts thousands of transposons, which probably contributed to the scrambling of its genome, either through actual transposition or via ectopic recombination between transposons of the same family. Unlike domesticated transposases in other eukaryotes, micronuclear transposons display evidence of purifying selection acting on their encoded proteins [23,54] and may still be active outside the control of the host cell. The presence of active transposons in the micronucleus may have provided the selective pressure for acquisition of a template-directed genome unscrambling system as part of macronuclear development [55] as a mechanism for promoting the long-term stability of the genome and robustness to perturbations.

Recent discoveries reveal that micronuclear transposons play a surprisingly direct role in both macronuclear development and genome rearrangement [23]. Micronucleus-limited transposase genes are expressed during macronuclear development, but silent during vegetative growth. The experimental silencing of these transposases by RNAi results in aberrant unscrambling patterns in the macronuclear genome, suggesting that transposons play an active role in genome rearrangement. It is possible that the nanochromosome templates are composed of RNA to protect the developing macronucleus from the integration of active transposons. In this regard, *Oxytricha* seems to have avoided the deleterious effects of internal transposon activity through template-directed genome rearrangement that, itself, employs the transposon proteins. Thus, the properties of nuclear dimorphism and template-directed macronuclear development in *Oxytricha* demonstrate the principles of spatial separation and metabolic incorporation that are thought to make early replicating systems resistant to selfish replicators.

Concluding remarks

Here, we have discussed the nuclear dimorphism and genome structures of *Oxytricha* to demonstrate several plausible dynamics of early genetic systems during the transition to modern genomes. *Oxytricha* is not by any means a ‘living fossil’, given that its phylum, Ciliophora, is both eukaryotic and not particularly deep branching. However, by analogy we have used *Oxytricha* to introduce several new hypotheses about early genomes. We invoke the process of template-directed genome rearrangement in *Oxytricha* to model an evolutionary landscape in which protein polymerases could evolve gradually from ligases. We have also observed that the dynamics of *Oxytricha* amitotic macronuclear division suggest that unmanaged cell division in early life could be viable if hereditary molecules were present in high copy-numbers. Finally, we employed observations of lateral gene

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transfer and active transposon mediation in *Oxytricha* to improve our understanding of the consequences of genome instability for early life. Although the particular genomic traits that we discuss are unique to *Oxytricha* and closely related genera, we encourage the further exploration of extant organisms, particularly those with atypical genetic systems [56], to help elucidate features of early cellular life.

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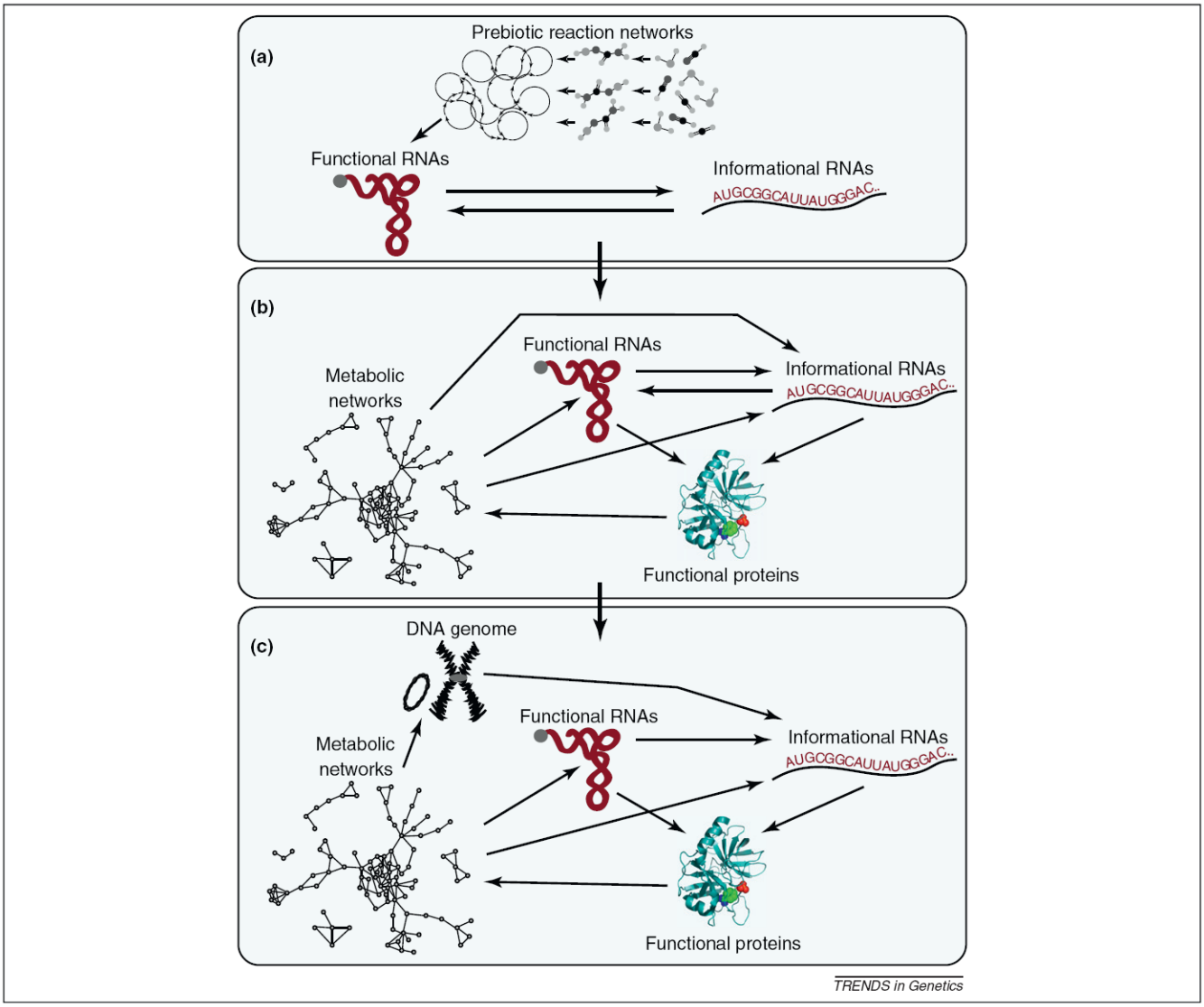

Figure 1.

The development of the modern genetic system from an RNA-dominated precursor genetic system. **(a)** The first genetic system probably involved informational RNAs encoding ribozymes which facilitated the replication of those informational RNAs [1]. Given the narrow catalytic range of ribozymes, this system probably relied on substantial networks of prebiotic chemistry to provide activated nucleotides [6]. **(b)** Protein synthesis by translation most likely arose from this RNA-based system [7] and rapidly developed into a highly processive, high-fidelity system [8]. Appropriately, the translation system is dominated by functional RNAs, including the ribosome itself, which has a ribozyme active site in its highly conserved core [57,58]. **(c)** The DNA genome probably arose from an RNA–protein precursor system. Deoxyribonucleotides seem to have been unavailable until the evolution of the ribonucleotide reductase protein enzymes [7]. Unlike translation, DNA replication and processing are dominated by protein functions rather than RNA functions, and core DNA-related functions do not appear to be universally conserved [10,11]. In the absence of significant bioinformatic evidence, the transition from an RNA genome to a DNA genome remains enigmatic.

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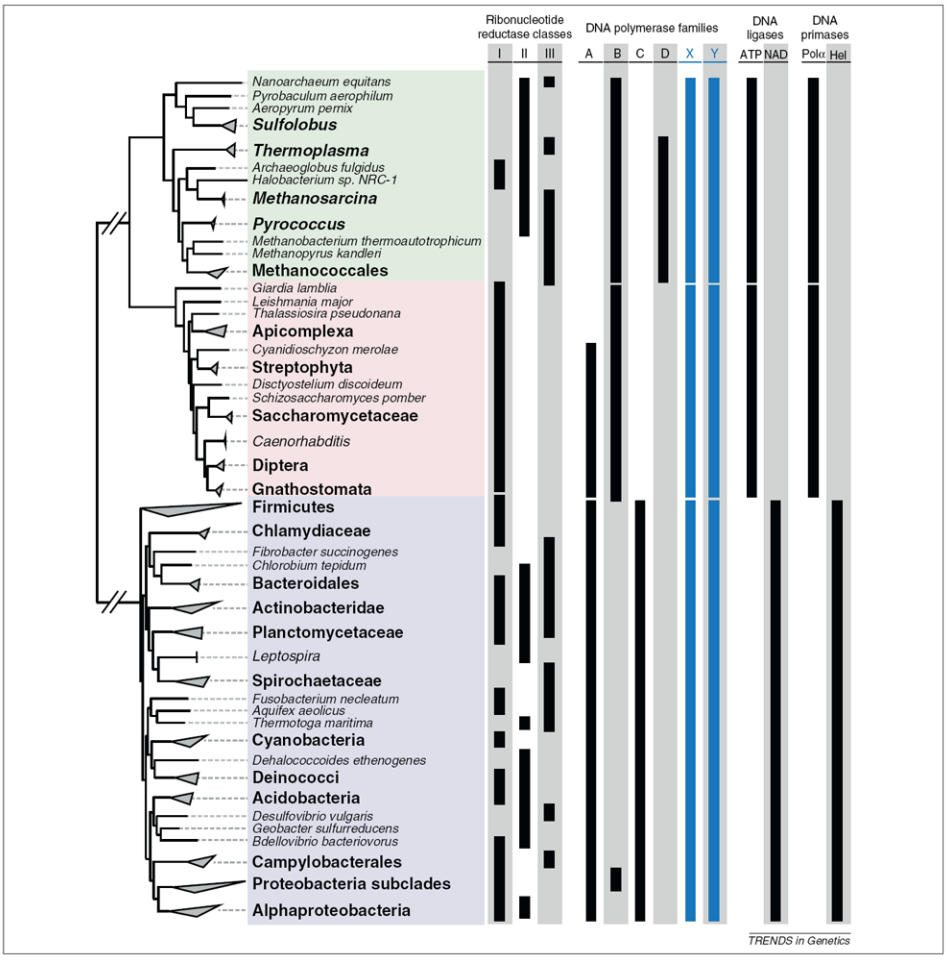

Figure 2.

A phylogenetic distribution of key enzymes involved in DNA synthesis. Unlike the protein translation system, very few features of DNA synthesis and processing are universally conserved. Ribonucleotide reductase is an enzyme required to produce deoxyribonucleotides from ribonucleotides. It is found in three distinct classes, I, II, and III, although ancient homology between them can be inferred from structural and mechanistic similarity. Six distinct families of DNA polymerases are known. None of the four standard DNA polymerase families (A, B, C, and D) has a universal taxonomic distribution. DNA polymerase families X and Y are universally distributed, but impart functions that are related to excision repair rather than DNA replication. The DNA polymerase X family catalyzes non-template-dependent DNA synthesis, while the DNA polymerase Y family polymerizes short segments across lesions. Bacteria use an ATP-dependent DNA ligase that is unrelated to the NADH-dependent DNA ligase used by Eukarya and Archaea. Similarly, Bacteria use a helicase associated DNA primase, whereas Archaea and Eukarya use a DNA polymerase a-associated DNA primase. The lack of a universally distributed set of enzymes involved in DNA synthesis suggests that modern pathways were still in the process of forming during the time of the last universal common ancestor (LUCA). Alternatively, DNA-related pathways may simply be more evolutionarily malleable than, for example, translation pathways, and this property would obscure their ancient phylogenetic signatures. The universal phylogenetic tree was previously generated in [59] and is based on 31 universal gene sequences from 191 genomes. The tree image was produced using the

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Interactive Tree of Life web server [60]. Clades representing groups of 25–40% similarity were collapsed to conserve space. Taxonomic distribution of ribonucleotide reductase enzymes were identified from the RNR database [61]. Taxonomic distributions of DNA polymerase families, DNA ligases, and DNA primases are extrapolated from [10,11], and do not represent a resolution capable of illustrating horizontal gene transfer. Ciliates are members of the Alveolata.

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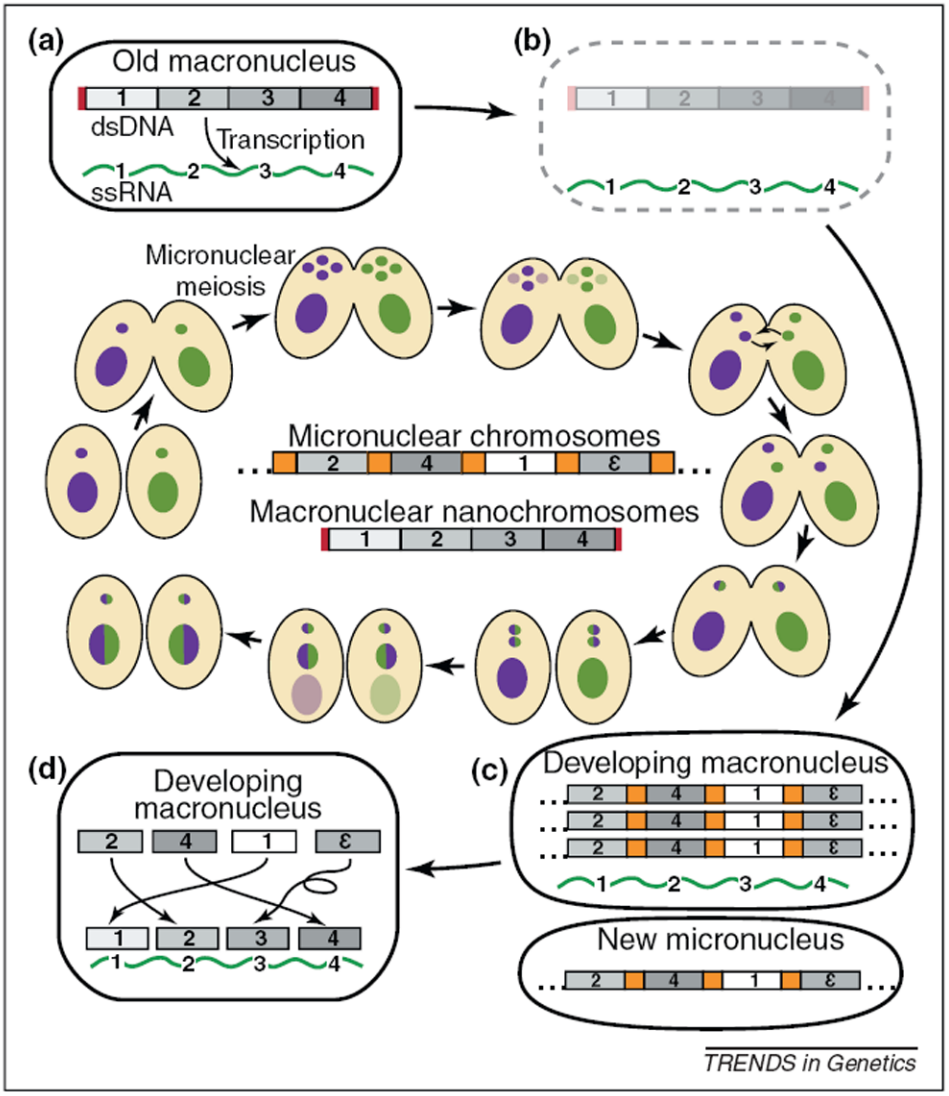

Figure 3.

A model for development of the *Oxytricha* macronuclear genome following conjugation. During conjugation (center) the micronucleus undergoes meiosis to produce four haploid nuclei, two of which exchange between partnering cells to form a new diploid micronucleus. During this process, the old macronucleus degrades and a new macronucleus differentiates from one copy of the new micronucleus [22]. The outer panels depict the process of macronuclear genome development by DNA rearrangement. **(a)** Conjugation triggers transcription of old macronuclear chromosomes into RNA. **(b)** The old macronucleus becomes dismantled, while the RNA transcripts of the chromosomes are retained and transported to the developing macronucleus. **(c)** The micronucleus replicates by mitosis and one micronucleus undergoes DNA amplification to produce material for the macronuclear genome. **(d)** Segments of micronuclear DNA (numbered 1–4) are reorganized using the macronuclear transcripts as a template for RNA-guided DNA rearrangement (including inversion of segment 3). Red bars indicate telomeres at the ends of nanochromosomes. Orange rectangles indicate deleted micronuclear DNA that separates DNA segments retained in the macronucleus.