

Adaptive mechanisms in pathogens: universal aneuploidy in *Leishmania*

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Genomic stability and maintenance of the correct chromosome number are assumed to be essential for normal development in eukaryotes. Aneuploidy is usually associated with severe abnormalities and decrease of cell fitness, but some organisms appear to rely on aneuploidy for rapid adaptation to changing environments. This phenomenon is mostly described in pathogenic fungi and cancer cells. However, recent genome studies highlight the importance of *Leishmania* as a new model for studies on aneuploidy. Several reports revealed extensive variation in chromosome copy number, indicating that aneuploidy is a constitutive feature of this protozoan parasite genus. Aneuploidy appears to be beneficial in organisms that are primarily asexual, unicellular, and that undergo sporadic epidemic expansions, including common pathogens as well as cancer.

The flexible genomes of *Leishmania*

The discovery of chromosomes as carriers of DNA and the visualization of karyotypes have greatly improved our understanding of how genetic material is organized. Most eukaryotic organisms are diploid, in other words two copies of each chromosome (homologs) are present. Alterations in chromosome number – either polyploidy or aneuploidy (see [Glossary](#)) – are usually associated with developmental abnormalities. Polyploidy, where the entire set of chromosomes is multiplied, is relatively common in plants and occurs occasionally in invertebrates, fishes, and amphibians [1]. Moreover, ancient whole-genome duplications have shaped the genomes of a wide range of eukaryotes, such as yeast [2], plants [3], and vertebrates [4,5]. Thus, polyploidy is an important contributor to the evolution and diversification of many organisms. By contrast, chromosome gain or loss (aneuploidy) in multicellular eukaryotes is either lethal or results in severe abnormalities, for example trisomy of chromosome 21 in humans with Down syndrome [6]. Despite the predominantly negative effects of aneuploidy, supernumerary chromosomes have been reported in tumor cells, yeast, and recently the protozoan *Leishmania* [7–11].

Leishmania (Kinetoplastida, Trypanosomatidae) is a genus of single-celled parasites transmitted between human hosts by sand flies, causing leishmaniasis, a debilitating

disease spread worldwide, characterized by a spectrum of clinical forms [12]. With no vaccines available, leishmaniasis is a major global health problem due to the emerging resistance against the first-line drugs and limited availability of alternatives [13].

Since the publication of the genome sequence of *Leishmania major* isolated in Israel [14], references were also created for a Spanish *Leishmania infantum* [15], and the genetically distant *Leishmania braziliensis* [15]. Recent advances in next-generation sequencing technologies boosted this research and provided a reference genome for other *Leishmania* species: a Nepalese *Leishmania donovani* [16], a *Leishmania mexicana* strain from Guatemala [17], as well as an African lizard-infecting sample, *Leishmania tarentolae* [18]. Data for *Leishmania panamensis* and *Leishmania tropica* and close relatives of *Leishmania*, *Endotrypanum* and *Crithidia*, are also available (www.ncbi.nlm.nih.gov/sra; www.tritrypdb.org). With the continued sequencing of other Trypanosomatidae species, these investigations contribute to our understanding of the complex biology of *Leishmania*, including its wide spectrum of pathologies, its capacity to persist in zoonotic as well as anthroponotic hosts and, most importantly, its mechanisms of drug resistance.

Old World *Leishmania* species (*L. donovani*/*infantum*, *L. major*, and *L. tarentolae*) have 36 chromosomes, whereas 35 are present in *L. braziliensis*, and 34 in *L. mexicana*, as result of unique chromosome fusion events [15,18–20]. Gene content and synteny are remarkably well conserved across the species in comparison to other microbes, despite an estimated divergence time of 20–100 million years [15]. In contrast to this degree of conservation, the sizes of

Glossary

Aneuploidy: genome state where the total number of chromosomes deviates from an exact multiple of the haploid chromosome number. This is the result of the loss or gain of one or more chromosome copies. Deletions or duplications of chromosome fragments result in partial aneuploidy.

Disomy: the presence of two copies of a given chromosome. In normal, diploid organisms, all chromosomes are disomic.

Euploid: having the normal number of chromosomes, in other words two copies of each chromosome in a diploid organism.

Hexasomic: having six copies of a given chromosome.

Karyotype: the number and appearance of all the chromosomes present in a cell.

Monosomy: one copy of a given chromosome. This is the ground state for chromosomes of haploid organisms, such as some yeast strains. In diploid organisms, this is the result of the loss of one chromosome copy.

Polysomic: having multiple copies of a given chromosome.

Tri- or tetrasomy: the presence of three or four copies of a given chromosome.

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Keywords: aneuploidy (experimental and natural); *Leishmania*; genome instability; fitness; population genetics; recombination; clonality.

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homologous chromosomes are highly polymorphic [7,21–24]. This size variation is probably the result of DNA amplification and deletion events, mainly in the subtelomeric repeat regions [7,25]. Although *Leishmania* is considered to be a diploid organism, supporting evidence is not abundant [22]. Several lines of evidence suggest that all *Leishmania* species are naturally aneuploid [16,17,26].

In this review we examine the origin and species distribution of aneuploidy in *Leishmania* and its relevance to other pathological agents. Evidence for aneuploidy in *Leishmania* species is explored, as well as its frequency on individual chromosomes across species, within populations and in single isolates with respect to modern leishmaniasis treatment regimes. Understanding how chromosome copy number changes can be beneficial provides a foundation for future work on tackling the evolution of aneuploidy-driven pathogenicity and drug resistance.

Emergence of aneuploidy in experimental *Leishmania* strains

Additional chromosome copies have been observed in laboratory *Leishmania* strains when attempting to construct gene knockouts or after induction of drug resistance. The generation of null mutants by homologous recombination has proven unsuccessful for the *dhfr-ts* gene (dihydrofolate reductase – thymidylate synthase) in *L. major* [27], *crk1* (a *cdc2*-related kinase) in *L. mexicana* [28], trypanothione reductase in *L. donovani* and *L. major* [29], and LmjF.01.0750 (a putative protein kinase) and LmjF.01.0760 (a hypothetical protein of unknown function) in *L. major* [30]. A copy of the targeted gene was always present as a result of chromosome duplications (tri- or tetrasomy), whole-genome duplication, or translocation of the chromosome fragment carrying the target gene. Aneuploidy was also observed in laboratory strains of *L. major* and *L. infantum* after induction of drug resistance: transcriptional profiling using microarrays identified altered expression of genes on six different chromosomes in both species [10,11]. Quantitative Southern blot and comparative genomic hybridizations confirmed that the variation in expression corresponded to changes in the copy number of these chromosomes. Remarkably, the chromosomes reverted to disomy in the absence of drug pressure, which indicates that aneuploidy was not stable and could assist drug resistance. Other experimental stresses such as subcloning could contribute to chromosomal polymorphism [9], however, a recent report indicates that, in most cases, the chromosome ploidy pattern in clones is similar to that of the parental strain [26].

Aneuploidy in natural *Leishmania* populations

Since the errant karyotypes were first discovered, it was suspected that aneuploidy occurred in natural *Leishmania* strains; differences in karyotype have been found in *L. infantum* isolates and derived clones [7,22]. The number of homologous chromosomes is also polymorphic. For example, although trisomy was observed at chromosome 1 in *L. major* strain Friedlin [23,25], this was not always found in other studies [17].

Recent analyses of *Leishmania* genomes have shown that aneuploidy is more widespread in this genus than previously assumed (Figure 1). The chromosome copy

numbers of two strains of *L. major*, *L. mexicana*, *L. donovani*, and one strain of *L. braziliensis* and *L. infantum*, were computed from genome-wide read depth coverage and showed the presence of supernumerary chromosomes in all samples: a mere twelve chromosomes were disomic in all samples [17]. *L. braziliensis* was predominantly triploid with some tetrasomies and one hexasomic chromosome, whereas the other strains and species had a more variable ploidy.

However, most strains used in these studies have been maintained in culture for many years and hence may not be representative of the species, and greater insights can therefore be drawn from aneuploidy surveys in natural populations. This question was addressed by another investigation that sequenced the genomes of 17 *L. donovani* lines derived from clinical samples from India and Nepal and used read depth analyses to reveal extensive aneuploidy in each line [16]. Only 9 (including 7 of the chromosomes found to be disomic [17]) of 36 chromosomes were disomic in all 17 samples, and 1 (chr31) was tetrasomic in all strains tested so far; the copy number for the latter has been reported as more than two in all sequenced *Leishmania* species and strains. The 26 remaining chromosomes all had variable copy values across the different lines. Consequently, each isolate had a unique karyotype. This stunning level of chromosome copy number diversity was observed in a genetically monomorphic population where only 0.011% of individual nucleotides varied [16]. This finding of aneuploidy in natural populations refutes the hypothesis that changes in chromosome number are merely an artifact of long-term maintenance of the parasite in culture.

Chromosome copy numbers calculated from the depth of mapped reads have shown intermediate values (e.g., between 2 and 3) that may be a consequence of a mixed cell population with disomic and trisomic chromosomes within the isolate sample. This chromosomal mosaicism was demonstrated by fluorescence *in situ* hybridization (FISH) analysis of seven chromosomes in *L. major* isolates and derived clones [26]. None of these chromosomes were exclusively disomic; they were all monosomic, disomic or trisomic. Thus, aneuploidy appears to be the norm in *Leishmania*, to the extent that an isolate or a clone consists of a population with mixed ploidy. Although the existence of variable copy cells has only been verified in *L. major*, intermediate chromosome read depth values in other species suggest that this is frequent in *Leishmania* [16,17]. The same is true for the related parasite *Trypanosoma cruzi* that has extraordinary diversity within hosts [31]. Comparative genomic hybridization of different *T. cruzi* strains has illustrated frequent variation in chromosome copy numbers [32].

Aneuploidy is usually disadvantageous

Deviation from the euploid chromosome number is associated with developmental defects in a wide range of organisms such as plants, flies, and humans, and in some unicellular eukaryotes. Aneuploidy is detrimental to cellular fitness and interferes with cell proliferation [33], most probably due to stoichiometric imbalances in essential proteins resulting from changes in gene dosage (reviewed

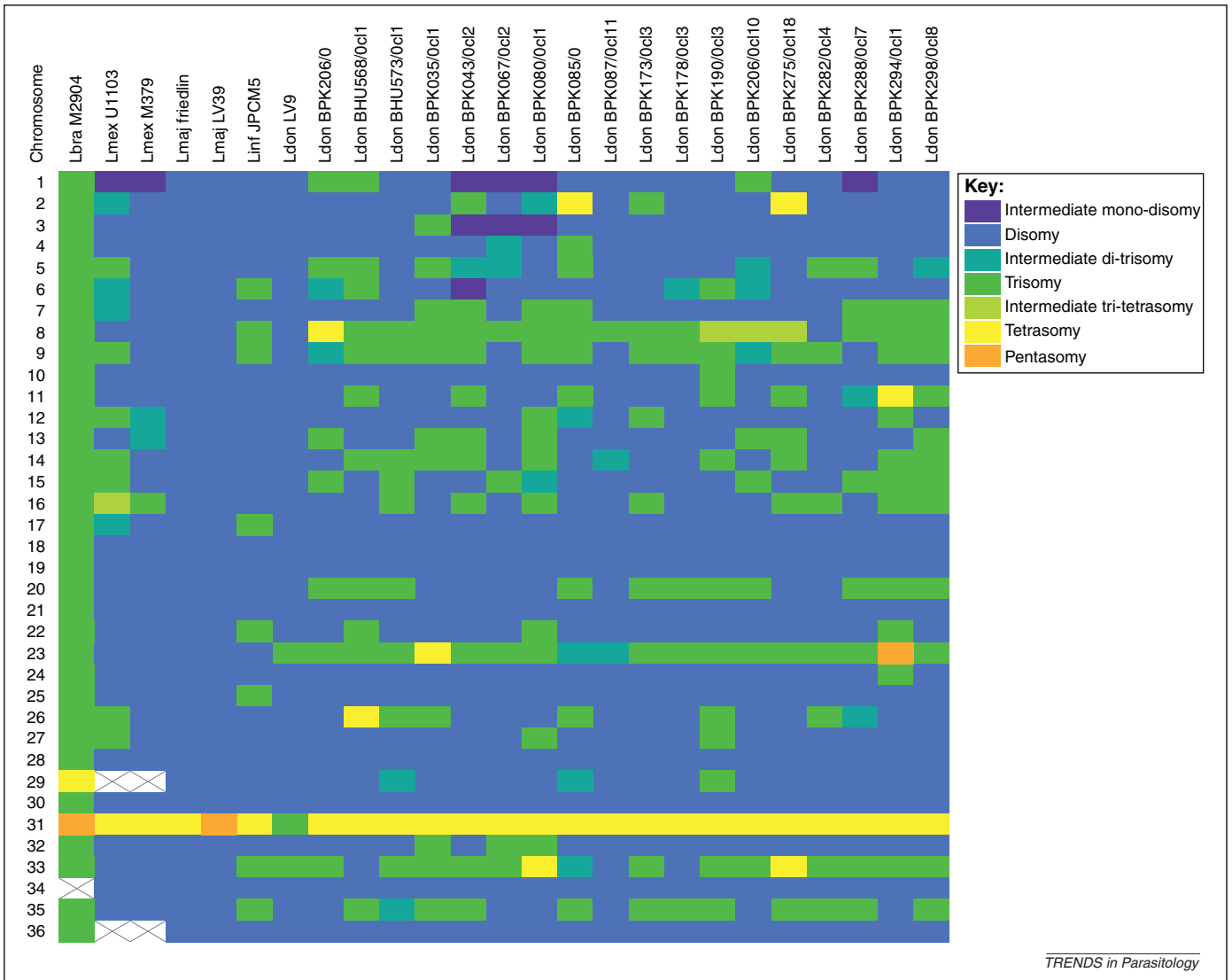


Figure 1. Aneuploidy in different *Leishmania* strains. The copy numbers of each chromosome as determined by read depth analyses of the genome sequences are represented by the different colors (blue, disomic; green, trisomic; yellow, tetrasomic; orange, pentasomic). Intermediate colors represent intermediate read depth values, which are probably caused by mixed cell populations with chromosomal mosaicism. Some data from the first eight strains are taken from Rogers *et al.* [12], and the chromosome copy numbers of the remaining 17 strains are from Downing *et al.* [11]. The crossed boxes indicate non-existing chromosomes as a result of fusions between chromosomes 20 and 34 in *L. braziliensis* and between chromosomes 8 and 29 and chromosomes 20 and 36 in *L. mexicana*. Abbreviations: Lbra, *Leishmania braziliensis*; Lmex, *Leishmania mexicana*; Lmaj, *Leishmania major*; Linf, *Leishmania infantum*; Ldon, *Leishmania donovani*.

in [34]). In yeast, the effects of additional chromosomes are relatively well studied. Aneuploid budding yeast (*Saccharomyces cerevisiae*) strains have a proliferative disadvantage and altered metabolism compared to normal cells as evidenced by increased glucose uptake and by sensitivity to conditions that affect protein homeostasis [35]. Such yeast strains experience increased chromosome loss, mutations, mitotic recombination, and defective DNA damage repair, highlighting that aneuploidy is not only a consequence but also a cause of genomic instability [36]. However, under specific circumstances cells 'benefit' from an aneuploid karyotype, and significantly this is predominantly found in clonal populations such as cancer cells as well as unicellular eukaryotes. Most solid tumors are aneuploid – approximately 25% of cancer cell karyotypes are affected [37], despite the apparent negative effects that antagonize cell proliferation [38]. Whether this aneuploidy is a source or a byproduct of tumorigenesis is still unclear. Nonetheless, proliferation would be promoted by elevated oncogene

copy number or decreased tumor suppressor gene copies [6,39–41].

Why does *Leishmania* tolerate aneuploidy?

If aneuploidy is generally so harmful, why is *Leishmania* able to tolerate or even benefit from the presence of extra chromosomes? Aneuploidy appears to be ubiquitous and thus may be an essential feature of *Leishmania* [26]. Genomic plasticity, including mosaic ploidy and the maintenance of aneuploidy, is a source of diversity in the absence of sexual recombination [26,42]. Although *Leishmania* was thought mainly to reproduce clonally [43,44], this assumption is now challenged by population-level analysis [45] and by the demonstration of genetic recombination in the fly vector [46]. Nevertheless, aneuploidy can be considered as a non-Mendelian means to create genomic variation when recombination is absent or minimal. It may also regulate gene dosage – as in other trypanosomatids, the regulation of gene expression does not take place at

transcription, and instead *Leishmania* relies on post-transcriptional degradation of mRNA, translational control, and/or protein modification and degradation [47,48]. Elevated gene expression may be accomplished before transcription by gene amplification through duplication, the formation of extra-chromosomal amplicons, or chromosome duplication. Correspondingly, chromosome loss may facilitate the downregulation of specific genes. A correlation between post-transcriptional dosage and chromosome copy number has been reported in aneuploid yeast, cancer, mammalian cells, and one *L. donovani* line [16,33,49–51]; consequently, this needs to be tested more widely. However, the occurrence of tandem arrays of identical or near-identical genes on disomic instead of on polysomic chromosomes in *Leishmania* is suggestive of gene dosage adjustment by both gene and chromosome duplications [17]. It is not known whether chromosome instability is driven by alteration of the expression of specific genes or entire chromosomes.

Mis-segregation and stochastic replication of chromosomes can alter gene copy number within a single generation and thus may enable the parasite to adapt quickly to dynamic host and vector environments. Although some ploidy changes have fitness costs [33,52,53], the absence of complete diploids in the *Leishmania* species and *L. donovani* natural population implies that some do not.

Leishmania is not the only microbe that appears to benefit from additional chromosomes. The common fungal pathogen *Candida albicans* displays a high level of genomic plasticity, including aneuploidy, affecting one to three of the eight chromosomes, depending on the strain [54]. This is apparently beneficial under selective pressure: for example, aneuploid *C. albicans* strains under drug pressure have an increased fitness [55]. In addition, the close relative *Candida glabrata* ($n = 14$) also has a variable karyotype and generates a novel chromosome in response to antifungal drug pressure [56]. Gain of an extra copy of one of the 14 chromosomes in another pathogenic fungus, *Cryptococcus neoformans*, influences its virulence and adaptation to the human host [57].

Aneuploidy probably helps the pathogen to adapt to the host environment. The sand fly gut and mammalian macrophages are the primary *Leishmania* microenvironments. These differ in pH, temperature, and nutrients, and therefore require substantial alterations in gene expression during parasite life cycle differentiation [9,58]. The cells undergo drastic changes in motility, shape, and metabolism when they alternate between hosts. There is a clear need to explore the extent to which aneuploidy aids the parasite to thrive in both environments as well as in other related hosts.

What is the origin of aneuploidy in *Leishmania*?

Genomic changes in aneuploidy-tolerating pathogens can occur at a rapid rate [54,59,60]. In *C. albicans*, aneuploidy can give an adaptive advantage in stressful conditions after only one or two cell divisions, and the original euploid state can be restored in one step when conditions are returned to normal [54]. Reversion to the normal state is also observed in *L. infantum*, where additional chromosomes disappeared after removal of drug pressure [11]. The

generation of aneuploid karyotypes could be achieved by different mechanisms.

One mechanism could be the defective segregation of sister chromatids during mitosis or meiosis, as observed in cancer cells [61]. Correct segregation of replicated chromosomes depends on proper attachment of the microtubule spindle apparatus to the kinetochores, and on the checkpoint signaling pathway regulating the separation of the sister chromatids [62]. Spindle apparatus formation and chromosome segregation are affected by arsenite [63], a metalloid related to antimony, which is widely used to treat leishmaniasis. A similar effect from antimonial treatment may lead to chromosome copy number changes in *Leishmania* [11]. The recent observation of chromosomal mosaicism in *L. major* suggests, however, that aneuploidy is not caused by chromosome segregation defects, but instead by a chromosome replication error [26]. Chromosome segregation mistakes have yielded asymmetric chromosome pairs with an even total chromosome number in *Trypanosoma brucei* [64], but this was not observed in *L. major*, where the total chromosome number in asymmetric sets was always odd, hinting at a chromosomal replication deficiency [26].

Another mechanism leading to genomic instability could be a defect in double-strand break repair, as observed in *C. albicans*, [42]. In trypanosomatids, double-strand breaks are repaired by homologous recombination (HR), which is also required for correct chromosome segregation during meiosis [65]. Although *Leishmania* is capable of having a meiosis-like cycle [46], it is rather unlikely that defects in HR are involved in the generation of aneuploids.

Alternatively, aneuploid cells may evolve from unstable tetraploid intermediates that subsequently have undergone chromosome loss, as has been proposed as a model of genetic evolution of cancer cells [66]. Tetraploidization can take place by endoreplication or by fusion of diploid cells. The parasexual reproductive cycle of *C. albicans* involves the mating of diploids, formation of tetraploids, and subsequent random chromosome loss, until a more-or-less – often aneuploid – diploid state is reached [67]. Whether this genome erosion occurs in *Leishmania* is unknown, but tetraploid intermediates have not been observed so far.

Practical consequences of fluctuating karyotypes

The universality of aneuploidy in *Leishmania* has consequences for research and experimentation. Although it is difficult to create homozygous knockouts of genes on variable chromosomes, the generation of additional chromosomes allows the testing of gene essentiality. Homozygous knockouts of essential genes are lethal; during knockout attempts, the function of these genes may be rescued by the creation of extra chromosome copies [27]. For non-essential genes, it is useful to verify the copy number of the chromosome before knockout, given the highly variable chromosome copy number in several *Leishmania* species.

The karyotype variability of *Leishmania* affects drug target discovery in terms of choosing pathways whose genes are on stable chromosomes and are outside structurally variable regions. Targeting genes on chromosomes with unstable copy number could lead to the formation of

supernumerary chromosomes, reducing the efficacy of the treatment and inducing resistance.

Aneuploidy also has an impact upon population genetics studies because most tests assume either haploidy or diploidy. Traditionally, co-dominant markers are chosen for their ability to detect heterozygous polymorphisms. However, most methods are not capable of determining allele frequencies within samples, and molecular markers therefore need to be selected on stable, disomic chromosomes. Heterozygosity is a standard diversity metric based on the number of mutations between samples. Assuming a molecular clock, the relative heterozygosity of polysomic chromosomes in a population sample depends on the density of mutations since the chromosome copy number change, thus providing the possibility to distinguish chromosomes with highly variable somy with reduced heterozygosity from those with more stable long-term polysomy with associated higher diversity.

Low heterozygosity can be the result of several factors involving evolution and reproduction – such as inbreeding in *L. braziliensis* [45] – and it can result from fluctuating chromosome copy numbers too. When a disomic chromosome with heterozygous alleles switches to trisomy, a major haplotype (two copies) and a minor haplotype (one copy) will be produced. Subsequent reversion to disomy with loss of the minor haplotype chromosome without crossing-over will result in the loss of heterozygous alleles [27]. The immediate loss of one of the major haplotype chromosomes should have no detectable effect, but if there is a sustained period during which mutations accumulate on all three chromosomes without recombination, the genetic distance between homologous chromosomes will increase: this is known as the Meselson effect [68]. Similarly, transient monosomy will also lead to a loss of heterozygosity [69].

Alternatively, if a tri- or tetrasomic state is stable for a longer evolutionary period during which recombination occurs between the homologous chromosomes, this could provide a selective advantage from a greater net chromosome length on which mutations can occur while still keeping the original gene copies [54]. In this scenario, reversion to disomy could cause perceived higher heterozygosity depending on the chromosomes lost and would also violate assumptions of a neutral molecular clock [70]. Therefore, results from population genetics studies of aneuploid organisms must be interpreted in light of the potential for an acceleration of polymorphism on stable polysomic chromosomes, and a loss of mutations in unstable polysomic or monosomic chromosomes.

By harnessing high-throughput DNA sequencing approaches we can now efficiently measure variation within a sample at each site and use genome-wide coverage distributions to infer chromosome copy number. Importantly, inferring chromosome copy number diversity for the population instead of for individual samples is likely to alleviate ascertainment biases [71–73]. As a result of increased sequencing, this removes the need for the time-consuming task of sample cloning for genetic tests. Moreover, the increasing scope to pool many barcoded samples together on next-generation platforms means that chromosome copy number estimation is an inexpensive and effective method for sample karyotyping [74].

Concluding remarks

The observation of several organisms exhibiting structural genome variability challenges the long-standing paradigm that genome stability is necessary for normal development of eukaryotes. Genome instability and aneuploidy are prevalent in different organisms, such as trypanosomatids and yeast, and in others are drivers of disease such as cancers. Why these organisms are able to tolerate aneuploidy is yet unclear, but in the case of *Leishmania* the haploid, small genome (~32 Mb) is distributed over a large number of chromosomes, and accordingly altering the copy number of one chromosome does not affect many genes. This tolerance to aneuploidy makes *Leishmania* a good model in which to study genome instability and ploidy changes.

The presence of aneuploidy in several strains of *Trypanosoma cruzi* as well raises questions about its evolutionary origin. When did it arise? Did it evolve independently in both lineages, or is there a common origin? In case of the former, is it a feature of intracellular parasites specifically (both *T. cruzi* and *Leishmania* spp. infect cells and transform to amastigotes), or do extracellular trypanosomatids also exhibit it? To address this issue, the karyotypes of more pathogenic and non-pathogenic *Leishmania* and related species, such as *Crithidia*, *Leptomonas*, and *Endotrypanum*, need to be investigated.

Aneuploidy is relatively frequent in eukaryotic pathogens and may be a mechanism to adapt to the host environment and prevailing drug pressure. In *Candida*, *Leishmania*, and cancer, aneuploidy is often associated with drug pressure: whether it would also occur in the absence of drugs should be tested. In *L. donovani*, for example, extreme chromosome copy number variation may be the result of continuous drug pressure due to the treatment of infected patients between whom the parasites are transmitted. Therefore, there is a need to investigate whether extensive aneuploidy is also present in zoonotic species, such as *L. braziliensis*, in which the drug pressure is lower. This could be paralleled by testing why particular aneuploid *Candida* species are less pathogenic than others, to determine if aneuploidy is a key driver of virulence in hosts [75,76].

Although the potential ability of pathogens or cancer cells to acquire drug resistance by adjusting their chromosome copy numbers poses significant challenges for public health, the aneuploidy mechanism represents an ideal drug target. Identifying genes causing changes in chromosome number and understanding the associated effects are pivotal for effective drug development. Unwinding the genetics of genomic plasticity will be necessary to understand the evolution of both tumors and pathogens, and how they acquire resistance to drugs.

Acknowledgments

We thank Matthew Berriman for critical reading of the manuscript. We gratefully acknowledge financial support from the European Commission (grant number EC-FP7-222895, Kaladrug-R), the Flemish Ministry of Sciences (ITMA SOFI-B GeMInI), and the Baillet-Latour Foundation.

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