The Evolution of Mitochondrial Genomes in Fungi

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Abstract

Fungal mitochondrial (mt) genomes (mitogenomes) are diverse and highly variable both at the inter- and intra-species level. While they contain a certain set of conserved genes, exceptions with respect to gene content and order may be found among and within species of all phyla across the kingdom of fungi. Phylogenies based on the concatenated matrix of the conserved mitochondrial protein-coding genes are robust and at least as informative as the ones provided by nuclear-based gene matrices, irrelevant to their matrix sizes. The diversity of mitogenomes' size and structure, as well as presence (or not) of accessory elements (introns, ORFs, and plasmids), provides strong information regarding the genome's evolution and to an extent the evolution of the organism. This diversity is also evident in their variable gene order (synteny). Gene shuffling is common among the mt genomes of fungal species, even of closely related ones, like those belonging to the same taxonomic order. Recombination and horizontal gene transfer (HGT) events are among the major mechanisms involved, although there are several cases of gene shuffling which are the result of plasmid integration within the genome, intron mobility, and transposition. Nowadays, analyses of mitogenomes in fungal species provide evidenced insights related to the endosymbiotic event which led to the genesis of the mitochondrion in the proto-eukaryote and its evolution. This is due to the different evolutionary divergence rates that fungal mitogenomes present, given their rates differ from the known respective ones in metazoan and plant mt genomes. In this chapter, all aspects of fungal mitochondrial evolution are described, summarizing the existing knowledge on fungal mitogenomic structure, evolution, and dynamics.

Keywords

Mitochondrial genomes · Synteny · Structure of mitogenomes · Introns · Homing endonuclease genes (HEGs) · Plasmids · mt genetic codes · mtRNA polymerase · Phylogeny · α-proteobacterial endosymbiont

3.1 Introduction

The accumulation of the available whole genome sequencing (WGS) projects of fungal species has

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progressed exponentially over this last decade (Smith 2016). Among the sequences created by the analyses of these projects for the species under examination, a small sized genome (compared to the nuclear counterpart) is frequently assembled, the mitochondrial (mt) genome or mitogenome that is located within the mitochondria of the organisms. Even before the WGS projects, analyses of mitogenomes are of great interest to the scientific community for many different reasons (Smith 2016; Rubinoff and Holland 2005). They were preferred firstly because they were easier to handle, since mitogenomes are multi-copied even within a single cell, smaller in size, and more conserved in their gene content than the nuclear chromosomes (Clark-Walker 1992). This is probably the reason why mt genomes were the first non-viral genomes to be sequenced and used in early genetic studies [e.g., for fungal mitogenomes like the ones of *Podospora anserina* (Cummings et al. 1990) and *Hansenula wingei* (Sekito et al. 1995)]. Secondly, many of the genetic exceptions known up to now, such as the high and variable copy number per cell, different genetic codes, self-splicing introns, high A/T content, missing genes, and various mutational and evolutionary rates depending on the organism that carries them (Moritz et al. 1987; Clark-Walker 1992; Christinaki et al. 2022), are found in fungal mitogenomes. Even small genes or regions present differences that may be useful for investigating genetic attributes, as well as evaluating the taxonomic, phylogenetic, and evolutionary status of the organisms that carry them (Hebert et al. 2003; Rubinoff and Holland 2005; Song et al. 2020; Liu and Wang 2021). In fact, mitochondrial genomic regions are among the most widely used and informative genetic markers for population and evolutionary studies (Smith 2016; Wolters et al. 2015; De Chiara et al. 2020).

Fungi present a diversity which is fundamentally informative for studying the evolutionary mechanisms that govern life. Within the kingdom of fungi, more than 3.3 million different species are expected to be discovered (Hawksworth and Lücking 2017), demonstrating species variability which is worth studying, given that fungi inhabit all global ecosystems and exhibit diverse modes of life, including saprotrophism, parasitism, and commensalism. Therefore, their genomic study is imperative for obtaining the required insights to explain evolution among other basic or applied scientific questions.

In this chapter, the acquired knowledge concerning the mitogenomes of fungal species will be presented under the prism of their contribution to evolution. Specifically, general features including genome content and size, gene order (synteny), intron abundance, intergenic regions' variability, genetic code usage, and mutational divergence rates will be discussed in depth, in respect of their contribution in the evolution of mitogenomes, as well as the evolution of the organisms. For reasons of consistency throughout this chapter, mt gene names will follow the nomenclature proposed by Boore (1999).

3.2 General Features of Mitogenomes

Fungal mt genomes present remarkable diversity in size, while their core gene content can be considered generally conserved. Their features are similar to the ones found in metazoa, but enriched with accessory elements like introns, additional genes, usually unidentified open reading frames or pseudogenes, plasmids, and large intergenic regions. The inclusion of these elements usually renders fungal mitogenomes larger than their metazoan counterparts (Lang et al. 1999). There are exceptions within early-diverging species belonging to Microsporidia and Neocallimastigomycota, which do not contain any mt genomes (Bullerwell and Lang 2005; James et al. 2013).

The typically conserved fungal mitogenome consists of ~30–40 genes implicated in the most important function of the cell, i.e., production of ATP through aerobic respiration. Specifically, there are genes that encode proteins functional as subunits for NADH dehydrogenase (*nad*1-*nad*6 and *nad*4L), apocytochrome b (*cob*), cytochrome c oxidase (*cox*1-*cox*3), and ATP synthase

(*atp*6, *atp*8, and *atp*9). The conserved package of fungal mitogenomes is completed with 2 genes encoding rRNA subunits, i.e., *rnl* and *rns* for the large (23S) and small (16S) rRNA subunits, respectively, a protein important to the assembly of the small ribosomal subunit, *rps*3 (in the early literature it is also called *var*1), a variable number of ~22–26 tRNA *trn* genes, which usually cover all tRNA needs of the protein synthesis within the organelle, and tRNA processing genes, like *rnp*B (Cummings et al. 1990; Clark-Walker 1992). There are, however, several fungal mitogenomes which lack a varying number of these core genes. For instance, the mt genomes of species belonging to Cryptomycota, Schizosaccharomycetaceae, Saccharomycetaceae, and Saccharomycodaceae lack the *nad* genes (Bullerwell et al. 2003; James et al. 2013; Freel et al. 2015; Quandt et al. 2017; Christinaki et al. 2022). Additionally, the mitogenomes of most Chytridiomycetes lack many *trn* genes (Forget et al. 2002; van de Vossenberg et al. 2018), while several mitogenomes of taxonomically dispersed species lack one or two genes, like *rps*3 (Bullerwell et al. 2000; Korovesi et al. 2018), *atp*8 and *atp*9 (Franco et al. 2017), or *trn* genes, such as *trn*A (for $tRNA^{Ala}$) and *trn*C (for $tRNA^{Cys}$) in *Lecanicillium muscarium* (Kouvelis et al. 2004) (see Table 3.1).

Other genes, which often enrich the mitogenomes' gene content, are the ones encoding for (a) homing endonucleases (HEs) or reverse transcriptases (RTs) needed for horizontal mobility of themselves (and usually of the mt introns which carry them) and (b) even less frequently, DNA and RNA polymerases or remnants of these genes and (c) unidentified ORFs with either an unidentified function or a role in fungal pathogenesis (see corresponding sections below).

Studies of genome sequences show that DNA transfer from organelles to the nucleus occurs at very high rates and that this influx is an ongoing process, occurring since the first endosymbiotic event (Timmis et al. 2004). Still, questions related to the reasons for which the few genes of fungal mitogenomes comprising its main core remained "captive" within the organelle, when the first bacterial endosymbiont turned out to be the mitochondrion (Adams and Palmer 2003), remain unclarified and addressed with suggested hypotheses. These core genes were not even transferred during the much later organellar gene transfer (OGT) incidents, which include the occasional transfer of large DNA genes/fragments from the mitochondrion to the nucleus and, in rare cases, vice versa (Martin 2003). The main arguments that try to explain this hotly debated issue are (a) the protein structure of these genes' products or (b) the mitochondrion's need for redox control. The first theory, called hydrophobicity theory, supports that these genes remain within the mt genome because their products cannot be transferred from the cytoplasm to the inner mitochondrial membrane due to their amino acid content and structure. The hypothesis that these proteins remained due to their transmembrane structure seems plausible and also acceptable for the fungal mt genomes (Adams and Palmer 2003; Daley and Whelan 2005). The second theory supports that these genes had to be maintained and not transferred to the nucleus, due to their implication in the organelle's redox balance (Allen 2003). Thus, this core of genes can be found in all eukaryotes, including fungi and metazoa. Findings show that most of the hydrophobic proteins of both mitochondria and chloroplasts are encoded by the nucleus and transferred to the organelles, a fact that conflicts the hydrophobicity theory (Allen 2003). Mitogenomes, however, are highly compact in metazoa (sizes usually ranging from 17 to 26 kb), whereas in fungi the respective range is from 11,223 bp (*Hanseniaspora pseudoguilliermondii*) to 272.2 kb (*Morchella importuna*) (Liu et al. 2020b). In other words, if the evolution of mitogenomes was studied by focusing only on metazoa, the conclusion would have been that mitogenomes shrink in size during evolution. However, as it has recently been shown in a few studies of fungi and more specifically in species of the subphylum Saccharomycotina of the phylum Ascomycota (Christinaki et al. 2022; Hao 2022), the size of mitogenomes changes without a clear pattern of reduction or expansion, since both of these processes seem to have happened in many different

Table 3.1 Available mitogenomes of fungi in public databases (e.g., NCBI Nucleotide and Organelle databases), the species taxonomy, and other attributes of their mtDNA. The number of fungal genera and their species according to the taxonomic order to which they belong is provided. The range of tRNA (*trn*) genes in each order, as well as "missing" protein encoding genes, i.e., genes of ribosomal protein S3 (*rps*3), ATP synthase (*atp*6, 8, 9), NADH dehydrogenase (*nad*1–6, 4L), cytochrome c oxidases (*cox*1-3), and apocytochrome b (*cob*) subunits, are given as numbers and ticks, respectively. Ribosomal RNA genes are not shown as they are always present in fungal mitogenomes (The table is based on data acquired until May 2022 from the Kortsinoglou A.M., Myridakis A.I., and Kouvelis V.N.—manuscript under preparation)

							"Missing" genes[a]													
							atp			*nad*							*cox*			
Phylum	Subphylum	Class	Order	Genera	Species	No of *trn*s	6	8	9	1	2	3	4	4L	5	6	cob	1	2	3	*rps*3
Ascomycota	Pezizomycotina	Arthoniomycetes	Arthroniales	2	2	12,18			✓												
		Dothideomycetes	Botryosphaeriales	1	1	26		✓													
			Cladosporiales	1	3	26–28															✓
			Dothideales	1	1	26															
			Mycosphaerellales	5	6	25–38			✓	✓	✓	✓	✓	✓	✓	✓					✓
			Pleosporales	11	14	21–38		✓										✓			✓
			Venturiales	1	1	N/A[c]															
		Eurotiomycetes	Chaetothyriales	3	5	22–25										✓					✓
			Eurotiales	6	32	10–31		✓			✓			✓						✓	✓
			Onygenales	9	14	25–26															✓
		Lecanoromycetes	Caliciales	1	2	24–28			✓												
			Lecanorales	13	25	29–17		✓													✓
			Ostropales	1	1	26		✓													
			Peltigerales	4	7	21–29		✓	✓							✓					✓
			Pertusariales	1	1	29															
		Leotiomycetes	Erysiphales	4	5	24–26													✓		
			Helotiales	9	13	21–33		✓		✓	✓	✓	✓	✓	✓	✓				✓	✓
			N/C[b]	1	4	27–29															
			Thelebolales	1	1	17		✓		✓	✓	✓	✓	✓	✓	✓					
		Orbiliomycetes	Orbiliales	5	7	18–26															✓
		Pezizomycetes	Pezizales	3	3	14–31															
		Sordariomycetes	Diaporthales	9	4	25–28									✓	✓					
			Glomerellales	2	12	25–29						✓								✓	

			Hypocreales		121	18–30			✓	✓			✓	✓		✓	✓	✓	✓	✓	✓
			Magnaporthales		–	N/A															
			Microascales	5	12	25–31			✓												
			Ophiostomatales	3	6	24–27			✓												✓
			Sordariales	4	6	26–28			✓							✓		✓	✓	✓	✓
			Xylariales	4	4	25–32		✓							✓						✓
	Saccharomycotina	Saccharomycetes	Saccharomycetales	38	245	23–47[a]		✓		✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓
	Taphrinomycotina	Pneumocystidomycetes	N/C	1	8	24–28										✓					✓
		Schizosaccharomycetes	Schizosaccharomycetales	1	3	24–25				✓	✓	✓	✓	✓	✓	✓				✓	✓
		Taphrinomycetes	Taphrinales		–	N/A															
Basidiomycota	Agaricomycotina	Agaricomycetes	Agaricales	30	51	14–36				✓							✓			✓	
			Auriculariales	1	2	22,23														✓	
			Boletales	7	10	25–27														✓	
			Cantharellales	3	6	25–27															
			Gomphales	1	1	24															
			Hymenochaetales	9	10	25–30															
			Phallales	2	2	24															
			Polyporales	7	20	25–29															
			Russulales	6	16	24–27											✓			✓	
			Sebacinales	–	–	N/A															
			Thelephorales	1	2	25,28														✓	
			Tremellales	4	7	16–23														✓	
			Trichosporonales	1	3	14–25		✓	✓		✓	✓		✓	✓	✓	✓		✓	✓	
	Pucciniomycotina	Agaricostilbomycetes	Agaricostilbales	1	1	23														✓	
		Cystobasidiomycetes	Cystobasidiales	1	1	24															
		Microbotryomycetes	Microbotryales	1	2	24,31															
		Pucciniomycetes	Pucciniales	3	6	23–24		✓								✓					✓
		Microbotryomycetes	Sporidiobolales	2	3	23–28															
	Ustilaginomycotina	Exobasidiomycetes	Microstromatales	1	1	22														✓	
			Tilletiales	1	2	24		✓		✓	✓	✓	✓	✓	✓	✓					✓
		Malasseziomycetes	Malasseziales	1	9	22–25															

(continued)

Table 3.1 (continued)

Phylum	Subphylum	Class	Order	Genera	Species	No of *trn*s	"Missing" genes[a]													
							atp			*nad*							cob	*cox*			
							6	8	9	1	2	3	4	4L	5	6		1	2	3	*rps*3
		Ustilaginomycetes	Ustilaginales	4	6	21–22															✓
	N/C	Blastocladiomycetes	Blastocladiales	2	3	25–26		✓	✓												
		Chytridiomycetes	Chytridiales	1	1	7															✓
			Rhizophydiales	1	1	7															
			Spizellomycetales	1	2	8,12								✓							✓
			Synchytriales	1	2	5															✓
		Monoblepharidomycetes	Monoblepharidales	3	4	7–9															✓
		N/C	N/C	2	2	23															
	Glomeromycotina	Glomeromycetes	Diversisporales	1	2	22,25															✓
			Glomerales	2	7	20–26														✓	✓
	Mortierellomycotina	Mortierellomycetes	Mortierellales	1	1	26															
	Mucoromycotina	Mucoromycetes	Mucorales	5	5	24–25															✓
	Entomophthoromycotina	Entomophthoromycetes	Entomophthorales	1	1	23															✓
	Kickxellomycotina	Harpellomycetes	Harpellales	1	1	27															
	Cryptomycota (phylum)	N/C	N/C	2	2	4,23				✓	✓	✓	✓	✓	✓	✓					✓
	TOTAL MITOGENOMES OF DIFFERENT SPECIES:				762																

[a]"missing" = not found in at least one species of the order. Moreover, the lack of all genes within an order (e.g., in Mycosphaerellales) may be the sum of different "missing" genes in different species of the order

[b]N/C = Non-characterized taxonomic group

[c]N/A = Submitted mitogenome BUT Not Annotated

independent events (Christinaki et al. 2022). A similar study, but with only 25 mitogenomes under examination, had reached the same conclusion for the kingdom of fungi (Aguileta et al. 2014). Interestingly enough, size diversity working restlessly toward both directions, i.e., either to expansion or reduction, can be even monitored within the same species, as it was shown with *Saccharomyces cerevisiae* (De Chiara et al. 2020).

3.3 Topology: Structure of Mitogenome

Another intriguing feature of fungal mitogenomes is their morphology. Almost all bilaterian animal mitogenomes are found as circular molecules (Boore 1999), with a small, unique region called D-loop as the epicenter for starting their replication (Falkenberg 2018). In fungi, such a unique region has not been determined, but in *Saccharomyces cerevisiae* and its close relatives, several specific regions have been determined to act as origins of replication (ori), while in other yeasts, different modes of replication have been proposed, i.e., the rolling circle replication followed by template switching. Thus, it is suggested that ori elements are not a universal attribute in mt genomes of yeasts (Chen and Clark-Walker 2018). Therefore, even today, except for *S. cerevisiae* and some of its related species, the mechanism of mt genome replication is not fully and experimentally clarified. Mitogenome structure remains ambiguous regarding circularity or linearity in fungi, with a few experimentally substantiated exceptions (Zardoya 2020). As a common belief, mitochondrial genomes are mostly circular, but findings regarding the prevalence of linear fungal mt genomes are growing exponentially. The notion about fungal mitogenome circularity is still, rather wrongfully, retained for three logically sound facts: (a) beyond any doubt, the circularity of most mt genomes of bilaterian metazoa, like the human mitogenome, which historically was among the first to be fully studied (Menger et al. 2021), (b) the endosymbiotic ancestor of mitochondrion which was a bacterium with a circular genome (Williamson 2002), and (c) the circularity of the restriction maps of fungal mt genomes (Bendich 1993). Exceptions that experimentally verified the linearity of the mitogenome were found in early-diverging fungi, like the chytrids *Hyaloraphidium curvatum* (Forget et al. 2002) and *Synchytrium endobioticum* (van de Vossenberg et al. 2018), and in yeasts, either as multipartite linear array (e.g., *Saccharomyces cerevisiae* and *Candida* spp. Maleszka et al. 1991; Valach et al. 2011) or monomeric linear with inverted terminal repeats (e.g., *Pichia pijperi*—Dinouël et al. 1993; *Hanseniaspora uvarum*—Pramateftaki et al. 2006). Especially in yeasts, full analyses, employing pulse field gel electrophoresis (PFGE) and mapping of termini with exonucleases, provided further knowledge by discriminating the telomeric regions of the linear mitogenomes into two types of terminal structures: type I, with inverted terminal repeats containing a covalently closed single-stranded hairpin (Dinouël et al. 1993), and type II, with the inverted terminal repeats composed of tandem arrays of large repetitive units which end with an incomplete repeat unit possessing a 5′ single-stranded extension of defined length (Nosek et al. 1995). Mitogenome linearity can also be found in other eukaryotes, besides fungi, and it seems to be a trait acquired independently many times throughout evolutionary history assuming genome circularity of the α-bacterial endosymbiont. This is also confirmed by observations that genome structure is not related to species phylogeny, as closely related fungal species may have different mt genome structures (Nosek et al. 1998; Christinaki et al. 2022). The prevailing hypothesis for this morphological change of the mitogenome involves the integration of mobile elements including linear mt plasmids or transposons (Nosek and Tomáska 2003; Fricova et al. 2010).

Regardless of the genome's conformation, mitogenomes are clustered in different numbers in the mitochondria and are packaged and protected within proteins in a structure that is reminiscent of the chromosomal DNA form of bacteria which is in accordance with

endosymbiotic theory (Gilkerson et al. 2013). Further experiments would, however, have to be performed in order to decipher the in vivo conformation of fungal mitogenomes, and thus, at the moment, it is prudent to refer to it as a circularly mapped fungal mitogenome when there is no clear experimental evidence for the linearity of the genome examined.

3.4 Synteny (Gene Order) and Genetic Rearrangements

The content of the core genes in fungal mt genomes is generally conserved (with only a few exceptions in which one or more genes are missing, as previously mentioned). The gene order (or synteny) of fungal mt genomes, however, presents extraordinary plasticity (Kouvelis et al. 2004; Freel et al. 2015; Aguileta et al. 2014) (Fig. 3.1). Nowadays, thanks to the WGS projects and the advent of next-generation sequencing (NGS), the known mt genome sequences have increased exponentially, and it is evident that mitogenomic synteny is often so different from one species to another that its study should be considered a necessity if fungal mt genome evolution is to be analyzed. Synteny can be studied with two different approaches, considering (a) only the large sized core genes, i.e., rRNA and protein-coding genes, or (b) with the additional inclusion of smaller genes as the *trn* genes (coding tRNAs). The first approach offers the advantage of retrieving the order of the large, core genes in a simple depiction, which may provide information about ancestral core gene pairs or clusters (Kouvelis et al. 2004), with the additional retrieval of trans-spliced or divided genes, like the *rns* and *cox*1 genes of *Gigaspora rosea* (Nadimi et al. 2012), the *rns* of *Hyaloraphidium curvatum* (Forget et al. 2002), or of pseudogenes and chimeric genes [i.e., fused intronic homing endonuclease gene (HEG) with the preceding exon of the mt gene], like the ones found in the mitogenome of *Sclerotinia borealis* (Mardanov et al. 2014) and *Beauveria brongniartii* (Ghikas et al. 2010), respectively. However, the inclusion of *trn* genes offers more insights into the synteny and evolution of mitogenomes, because *trn* genes can be found either as single units, scattered among the large core genes, or as clusters, which are usually conserved when considering mt genomes of fungi belonging to the same order (Ghikas et al. 2006; Christinaki et al. 2022). Clear syntenic groups and patterns can be found among fungal species belonging to the same order (Fig. 3.1), especially in the case of Ascomycota (Pantou et al. 2008; Christinaki et al. 2022). Both types of distribution provide useful information under the prism of genomics and evolution. Single *trn* genes may play a dual role, either as termination signals of transcription (Schäfer et al. 2005; Varassas and Kouvelis 2022) or as hotspots for recombination events which may contribute to gene shuffling (Pantou et al. 2008). This latter theory has been proposed earlier for mt gene shuffling in metazoa (Saccone et al. 2002), but it must be used cautiously in fungal mitogenomes, since (a) species belonging to early-diverging fungi (EDF), like the ones belonging to Chytridiomycetes, usually contain a very small number of *trn* genes (Forget et al. 2002; Zardoya 2020) and (b) other repetitive elements like the GC islands of yeasts and *Neurospora crassa* may play the role of the target sequences for recombination (Yin et al. 1981; Dieckmann and Gandy 1987).

3.5 Mt Plasmids

The mt plasmids can be defined as sequences with little or no homology to the mitochondrial genome and have an independent evolutionary history that may render them intracellular parasites. They have been found in plants, fungi, and protists (Hausner 2012). Fungal mt plasmids are either circular or linear, usually carrying one or more genes involved in replication and transcription (DNA or RNA polymerases) (Griffiths 1995; Hausner 2012). They can be mainly discriminated into three different types: (a) linear or circular retroplasmids, which usually carry a gene that encodes a reverse transcriptase (Kennell and Cohen 2004), (b) linear plasmids

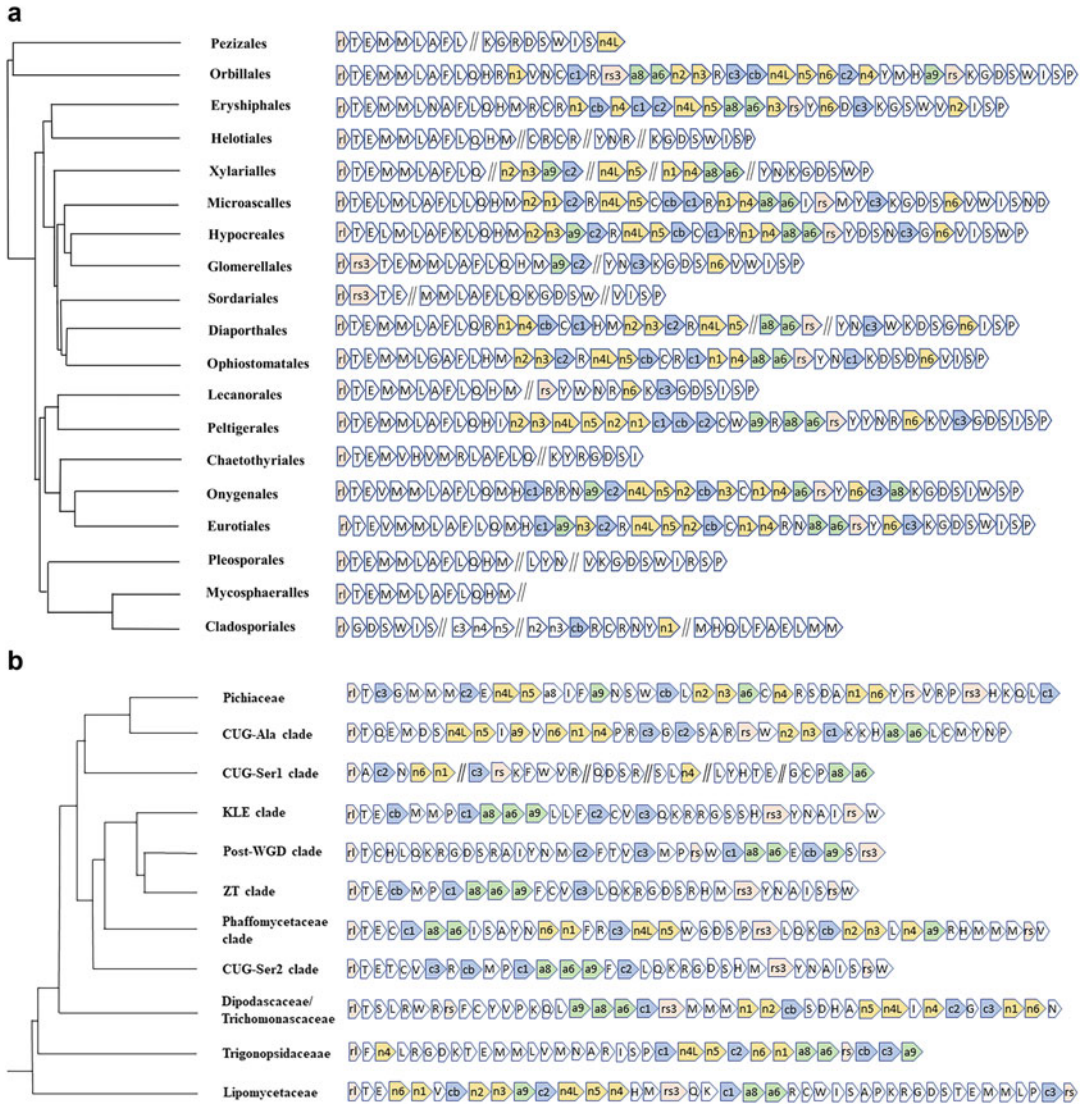

Fig. 3.1 Gene order of mitogenomes of species belonging to different orders of Ascomycota and their phylogenetic relationships: synteny of (**a**) various orders of Pezizomycotina subphylum and (**b**) the major phylogenetic clades of Saccharomycotina subphylum. Due to the variability of syntenic units among all members of each order, the sequence that represents most of the taxa of each order for Pezizomycotina and one representative from each clade of Saccharomycotina presented. Orders that contained less than three strains were not included in the analysis. Each arrow represents a gene. Pink arrows with rl and rs represent *rnl* and *rns* genes, respectively; blue and yellow arrows with cb, c1-c3, n1-n6, and n4L correspond to *cob*, *cox*1-3, *nad*1-6, and *nad*4L, respectively. White arrows with a single capital letter are for the *trn* genes which will encode the tRNAs of the respective one-letter designated amino acid. b) The double slash symbol (//) depicts a high variability of gene order at this part of the sequence. The dendrograms were based on the phylogenetic trees of Li et al. (2021b), Abdollahzadeh et al. (2020), and Christinaki et al. (2022) and represent the phylogenetic relationships of each order without showing distances

with terminal inverted repeats and with genes encoding either a DNA or an RNA polymerase, or both (Nakai et al. 2000; Klassen and Meinhardt 2007), and (c) circular plasmids that encode a DNA polymerase (Griffiths 1995). Linear plasmids, in particular, present the following usual features: (a) terminal inverted repeats, (b) genes for RNA and DNA polymerases of viral origin, and (c) termini with either covalently, hairpin-like closed ends (i.e., "hairpin" plasmids) or proteins which are covalently attached to their 5′ termini (i.e., "inverton" plasmids) (Meinhardt et al. 1997).

Their size varies usually from 5 to 10 kb for linear DNA plasmids, which encode for both DNA-dependent DNA and DNA-dependent RNA polymerases, or from 2.5 to 5 kb circular DNA plasmids that encode either a DNA polymerase or a reverse transcriptase (Cahan and Kennell 2005). It is highly intriguing that mt plasmids can be found either stably integrated in the mt genome, like in *Moniliophthora perniciosa* (Formighieri et al. 2008), or free and occasionally integrated in the mt genome, like in *Neurospora* spp. (Myers et al. 1989) and in *Podospora anserina* (Hermanns et al. 1994).

They have been considered as cryptic mitochondrial elements, since there is no evidence for a change in their host's phenotype (Hausner 2012), but according to several experimentally examined cases, they have also been correlated to senescence, life-span elongation, vegetative incompatibility, and reduced pathogenicity. Specifically, circular retroplasmids, like the *kalilo* or the *Maranhar* plasmids of *Neurospora intermedia*, may be inserted in several different domains of the mt genome and thus disrupt the function of essential genes, leading to growth cessation and aging, a process called senescence (Fox and Kennell 2001). However, in *P. anserina*, insertion of the linear pAL2-1 is correlated with an increased life span, and therefore, this mitochondrial plasmid has been named life-span-prolonging plasmid (Hermanns et al. 1994). Moreover, hairpin plasmids with variable hairpin loops have been correlated to vegetative incompatibility groups of *Rhizoctonia solani* (Katsura et al. 1997), while the circular plasmid pCRY1 of *Cryphonectria parasitica* is an infectious agent that reduces pathogenicity (Monteiro-Vitorello et al. 2000). Finally, in *Candida subhashii*, a linear invertron-type plasmid may have been domesticated to serve as its mitochondrial genome's telomeres (Fricova et al. 2010).

The potential of plasmid integration into their respective mt genome has not been fully resolved for all different types of mt plasmids, but plasmid-like sequences have been found to cluster in domains of mt genomes where low numbers of PstI palindromic sequences exist (Cahan and Kennell 2005). Short sequence homologies of the target regions within the terminal sites of the plasmid are necessary for the integration of the *kalilo* plasmid, whereas other integrated plasmids, like *Maranhar,* do not require a sequence homology for being incorporated in the mt genome. This renders plasmid duplications, horizontal plasmid transfer, or other unknown mechanisms as the proposed explanations for the existence and integration of mt plasmids into the mt genomes (Cahan and Kennell 2005). It is worth mentioning that in a few cases, like the mt genome of *Agrocybe aegerita*, there are two polymerase genes (*pol*B) flanked by two large, inverted repeats longer than 2.4 kb. Each repeat contains an identical copy of *nad*4 gene and, thus, this whole region is of linear-plasmid origin or might even be a remnant of a plasmid (Liu et al. 2020a). In *Moniliophthora perniciosa*, the existence of ORFs between the inverted repeats of the plasmid was considered as an indication of an integration-recombination event (Formighieri et al. 2008), despite the fact that no ORF coding for integrase activity has been found in mt plasmids of any fungal species so far (Cahan and Kennell 2005).

Overall, mt plasmids can be found quite often in fungal mitochondria, mostly in Ascomycetes and Basidiomycetes. Even if they have been integrated into the mt genome, they always carry at least a gene implicated in their function, usually a DNA or RNA polymerase. These genes share a common ancestor with their counterparts in phages (Pöggeler and Kempken 2004), but more recent horizontal gene transfer events cannot be excluded (Kempken 1995). In *M. perniciosa,* the

integration of the plasmid in the mt genome was a recent event, since the plasmid sequence is not adapted to the rest of the mt sequence, but the plasmid was not found in its close relative, *M. roreri* (Formighieri et al. 2008). The existence of the mt integrated plasmid might be an evolutionary advantage if the plasmid has been acquired recently and prolongs the life span of the organism carrying it (Formighieri et al. 2008). Similarly, in the mt genomes of *Glomus* spp., numerous DNA polymerase (*dpo*) sequences were retrieved with no similarity to each other, but they seemed to be related to *dpo* plasmid sequences in *Daucus carota*, a plant that may act as a host (Beaudet et al. 2013). This example is an indication that the plasmid integration or even the integration of plasmid-derived genes may occur repeatedly and independently throughout evolution. Additionally, the recent finding that mt plasmids are common in Agaricomycetes (with only the exception of *Cantharellus cibarius,* out of the 12 strains examined), but not in other known classes of Basidiomycota (Himmelstrand et al. 2014), indicates that vertical inheritance of mt plasmids and their genes from the common ancestor of Agaricomycetes may explain their existence with subsequent integration events into the mt genomes at many different independent occasions.

3.6 Nuclear–Mitochondrial Interactions

Nuclear mitochondrial interactions may be studied under the prism of three different approaches: (a) the involvement of nuclear (nc)-encoded proteins in the functions of mitogenomes, like replication, transcription, and DNA repair, (b) the mechanisms and processes that regulate the stoichiometry of nc- and mt-encoded products for efficient mitochondrial homeostasis, and (c) the exchange of nc- and mt-originated sequences harbored in both genomes. In brief, the fungal contribution to the evolution of these interactions to date may be presented as follows:

(a) Mt functions controlled by nc-encoded proteins—the example of mtDNA transcription

Functional processes of fungal mitogenomes rely on nc-encoded genes, as it has been shown in several studies, primarily in fungal model organisms like *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Schizosaccharomyces pombe* (e.g., Burger et al. 1985; Kleidon et al. 2003; Schäfer et al. 2005) and, recently, in other fungi like *Candida albicans* and entomopathogenic hyphomycetes *Beauveria bassiana* and *Metarhizium brunneum* (Kolondra et al. 2015; Varassas and Kouvelis 2022). While many related questions about the evolutionary establishment of these mechanisms remain unanswered, the study of the mt gene transcription in fungi for more than three decades provides certain well-substantiated hypotheses and brings insight into fungal mitochondrial genome evolution.

In specific, the finding of an eubacteria-like multisubunit RNA (msuRNA) polymerase in the mitogenome of a close relative of early-diverging amitochondriate eukaryotes, i.e., the protist *Reclinomonas americana* (Lang et al. 1997), as well as in fission yeasts (Seif et al. 2003) provided credibility to the theory that mt transcription was initially performed by a msuRNA polymerase which was subsequently lost and a nucleus-encoded single-subunit RNA (ssuRNA) polymerase was employed for the transcription of mt genes (Burger and Lang 2003). This replacement must have occurred early in the evolution, as a ssuRNA polymerase gene which resembled a T7 phage RNA polymerase gene has been identified in the early-diverging amoeba *Naegleria fowleri* (Cermakian et al. 1996). An alternative explanation to this is that the ancestral α-proteobacterium, which acted as the progenitor of the mitochondrion, was already infected with a lysogenic T7-like phage that had its genome integrated into the

bacterial one and that this phage-originated gene was transferred along with the majority of all the other bacterial genes into the nucleus (Varassas and Kouvelis 2022). The experimentally proven necessity of a sigma-like subunit (Mtf1 gene) for the infallible function of mt RNA polymerase Rpo41 in yeasts (Yang et al. 2015) adds further credibility to the latter theory.

Nevertheless, the transfer of genes from the endosymbiotic bacterial ancestor to the nucleus is beyond any doubt, as shown by the fungal mitogenome contents (see chapter above). The main mechanism proposed for the transfer of these genes to the nucleus is their transfer by mobile elements that employ the non-homologous (NHEJ) recombination for integration (Berg and Kurland 2000; Fonseca et al. 2021).

(b) Mechanisms of mitochondrial homeostasis

The existence of two independently originated protein products in almost all eukaryotic organisms, which have to interact and cooperate in order to achieve vital cell processes—including the production of ATP in mitochondria through oxidative phosphorylation (OXPHOS) and aerobic respiration in general—has recently been elucidated effectively through the study of yeasts and other organismal cells including mammalian ones (Youle 2019). In yeasts, as well as in the majority of other fungi, 14 (at maximum) different mt-encoded proteins have to interact with a median of 80 different nc-encoded counterparts, in order to assemble all the necessary complexes of OXPHOS (for a review, see Barros and McStay 2020 and references therein). Intriguing questions about the mechanisms and processes which are involved in the establishment of functional coordination of both mt- and nc-encoded proteins within the cell have only recently been partially addressed through comparative studies among yeasts and mammals (for reviews, see Isaac et al. 2018; Youle 2019). Therefore, in this subsection, the new advances based on the impact of fungi in deciphering their evolution will be presented in brief.

In a fungal cell there is usually one nucleus, while mitochondria and their mitogenomes are always larger in number (reaching in some cases 100 mitogenomes per cell; Burger et al. 2003). Therefore, a stoichiometry of the mt- and nc-encoded products is imperative. The balance of this stoichiometry is achieved through the coordination of RNA transcription in the nucleus with protein translation in the mitochondrial matrix and by degrading extraneous protein subunits (for a review, see Youle 2019). Proteolysis in the mitochondrial matrix (Liao et al. 2020), mitophagy and mitochondrial biogenesis (Pickles et al. 2018), and mitochondria-derived vesicles which directly target outer mitochondrial membrane proteins to lysosomes (Klecker et al. 2014) may act as additional backup processes. This may be achieved by eliminating excess proteins imported from cytosolic translation of nuclear genes or translated in the matrix from mtDNA-encoded genes that misfold in the absence of interacting partners from the alternate genome (for a review, see Youle 2019 and references therein). Moreover, when hydrophobic inner mitochondrial membrane proteins are overexpressed, they may clog the translocase of the outer membrane (TOM)/translocase of the inner-membrane (TIM) import channels and protect the cell through the unfolded protein response activated by the mistargeting of proteins (UPRam) and mitochondrial precursor overaccumulation stress (mPOS) pathways (Weidberg and Amon 2018). As for mtDNA-encoded proteins, mitochondrial translation machinery and translation itself (but not transcription) are markedly downregulated after clogging (Boos et al. 2019).

Thus, there are several processes which contribute to the maintenance of proteostasis and control of stoichiometry in nc- and

mt-encoded products. These mechanisms have also been found in mammals (Youle 2019). Therefore, there is strong indication of a common strategy that may have originated early in eukaryotic evolution and most probably at the stage of the last eukaryotic common ancestor (LECA.) That being said, a few other processes which help in the effective establishment of normal functional nc–mt interactions, like the mitophagy pathway, may be a later evolutionary acquisition (Youle 2019).

(c) NUMTs (NUclear sequences of MiTochondrial origin)

At present, it is well established that nuclear genomes in fungi, as well as in other eukaryotes, contain integrated fragments of mitochondrial DNA called NUMTs (NUclear sequences of MiTochondrial origin) (see, e.g., Kleine et al. 2009; Lafontaine et al. 2004). These usually correspond to pseudogenes or non-coding regions, but in a few cases, they have been incorporated within functional genes as exon patches (Noutsos et al. 2007). A comparative analysis of NUMTs in six different yeast species showed broad diversity in the number, size, and distribution of these elements within the chromosomes (Sacerdot et al. 2008). It is worth mentioning that in the examined yeasts the size of NUMTs was small (<400 bp), in contrast to the ones found in *Neurospora crassa* (the largest 4136 bp and average size of 647 bp—Richly and Leister 2004), and was distributed as either a tight mosaic (NUMTs overlap or are very close) or a loose one (more distant). In both cases, the mechanism of this mtDNA insertion into the nuclear genome may be considered a mutagenic phenomenon that may frequently inactivate a gene (Sacerdot et al. 2008). There are, however, NUMTs which have undergone a positive selection from the evolutionary aspect, since they have produced a new functional gene (Noutsos et al. 2007). Still, the mechanism of transferring NUMTs from the mitochondria to the nucleus is unresolved, but Beaudet et al. (2014) experimentally demonstrated the mobility of the *nad*1-*nad*4 intergenic region from the mitogenome of a *Rhizophagus irregularis* strain to its chromosomal DNA, thus suggesting an ongoing evolutionary process which may differ even among strains of the same species (not found in a second examined strain). Similarly, two *Trichoderma* strains harbored three NUMTs localized within the mitogenomic regions of *nad*5, *nad*6 and an intergenic region, while in the nuclear chromosomes they were found, as expected, in AT-rich regions which do not encode for any protein or RNA. It has been proposed that NUMTs have been implicated in increasing genetic diversity and facilitate genome evolution (Li et al. 2021a). Furthermore, it is experimentally proven in *Saccharomyces cerevisiae* and *Kluyveromyces lactis* that the NUMT acquisition and proliferation is a result of the existing DNA repair mechanism, either with the involvement of double-strand break (DSB) repair or the non-homologous end joining (NHEJ) pathway (Sacerdot et al. 2008).

3.7 Mt Gene-Based Phylogenies

Even from the early stages of molecular phylogenies, mt genes have been used as plausible alternative markers that may provide useful information in order to determine the phylogenetic relationships among fungal species, and to an extent their evolution. For example, studies have employed the genes of ATP synthase subunit 6 (*atp*6) (Kretzer and Bruns 1999) and of the small rRNA subunit (*rns*) (Pantou et al. 2005). When it became obvious that single gene phylogenies may be misleading in determining the phylogeny of whole organisms, these mt gene-based markers were used either in combination with nuclear and other mitochondrial genes as a concatenated matrix (Fahleson et al. 2004; Kouvelis et al. 2008; Theelen et al. 2021) or as a combined dataset of all the conserved mt genes

(Bullerwell et al. 2003; Kouvelis et al. 2004; Nadimi et al. 2016; Christinaki et al. 2022).

Mt-based phylogenies of the major lineages of fungi usually exhibit similar topologies to those produced by nuclear datasets (Bullerwell et al. 2003; Christinaki et al. 2022) with a few major differentiations (Pantou et al. 2008). One such "discrepancy" is the positioning of subphylum Saccharomycotina. Based on nuclear datasets, this subphylum is a sister clade to Pezizomycotina, and Taphrinomycotina are basal to both. Mt concatenated matrices produce phylogenetic trees showing the sisterhood of all yeasts (Saccharomycotina with Taphrinomycotina) and with Pezizomycotina as a sister clade of both (Bullerwell et al. 2003; Pantou et al. 2008; Freel et al. 2015; Christinaki et al. 2022). A plausible explanation for such phylogenetic incongruence is the different divergence rates of proteins and genes of mt vs nc origin. Recently, Christinaki et al. (2022) suggested a different molecular clock governing the evolution of the mt genes when compared with their nc counterparts. While the notion that mt genes evolve faster than nc genes present in metazoa is prevalent (Brown et al. 1982), it has lately been suggested that in plants and fungi, mt genomes exhibit a slower divergence rate (Sandor et al. 2018). However, Christinaki et al. (2022) proposed that fungal mt genes evolved slower than nc genes during the early phases of yeast evolution, and only recently has this changed so that mt genes evolve faster than their nc counterparts.

3.8 Intergenic Regions

As previously mentioned, introns and intergenic regions are some of the major determinants of fungal mt genome diversity. Intergenic regions vary greatly in fungal mt genomes in terms of both their size and content. Mitochondrial genomes of other eukaryotes, such as plants, may harbor larger intergenic regions, but fungi tend to have a very broad range of intergenic region size diversity. These regions in fungi may be as large as 88% of their whole mitogenome, as shown in the case of *Nakaseomyces bacillisporus* (Christinaki et al. 2022), while in certain cases they can also be as small as 1 bp, or non-existent, in cases when gene sequences overlap by 1 bp (Kouvelis et al. 2004). A characteristic example of such non-existing intergenic region is the case of *nad*4L and *nad*5 genes in several species such as *Lecanicillium muscarium*, *Neurospora crassa*, and *Podospora anserina*, where these genes overlap one another by a single base pair (Kouvelis et al. 2004). It has been suggested that intergenic regions may play an important role in genomic rearrangements and gene shuffling (Paquin et al. 2000). They often host repetitive sequences which can act as hot spots for recombination, as well as introns and ORFs with unknown functions (Paquin and Lang 1996; Liu et al. 2020b). This may explain the mobility of certain syntenic units, as there are indications of repeats in the intergenic regions that flank such syntenic units (Yildiz and Ozkilinc 2021; Zhao et al. 2021). It has been previously shown that the number of rearrangement events in fungal mitochondria is significantly correlated with the number of repeats in intergenic regions, the accumulation of which is mainly due to drift and mutation pressure (Aguileta et al. 2014). Similarly, small intergenic regions between genes may favor the formation and stability of syntenic units over time. Such an example can be observed in the case of *atp*6 and *atp*8 genes which are found as a conserved syntenic unit in most of the representative genomes of Pezizomycotina and Saccharomycotina, as well as in some species of Taphrinomycotina (Kouvelis et al. 2004; Christinaki et al. 2022), thus forming an ancestral conserved element. Additionally, small intergenic regions may help in the transcription of mt genes as fungal mt transcripts are initially polycistronic, but immediately after the formation of the polycistronic molecules, they mature to single cistrons as has been already shown in Ascomycetes (Breitenberger et al. 1985; Varassas and Kouvelis 2022). Therefore, repetitive elements in intergenic regions are most probably the result of stochastic duplication and recombinational events However, from the evolutionary prism, they can be involved in a diversity of functionally

relevant outcomes such as conversions of the mitogenome from linear to circular forms and vice versa (Valach et al. 2011), duplication of certain genes within the repeats (Theelen et al. 2021), and mobility through transposition (Paquin et al. 2000). The latter is further directly associated with the structure and organization of mitogenomes and, indirectly, with the regulation of the genomes' expression (de Zamaroczy and Bernardi 1986; Koll et al. 1996).

3.9 Introns and Intronic ORFs

Fungal mt genomes harbor various introns, in contrast to metazoa which rarely carry introns (Boore 1999). Based on their RNA secondary structure, sequence conservation, and splicing mechanisms, mt introns can be classified into Group I and Group II introns, but the former are more frequent in fungal mt genomes (Lang et al. 2007; Hausner et al. 2014; Sandor et al. 2018). Both types of introns have the ability to move within the genome by a self-catalyzation mechanism (for a recent review, see Mukhopadhyay and Hausner 2021). In order to do so, self-splicing introns of Group I usually contain homing endonuclease genes (HEGs), coding for homing endonucleases (HEs), i.e., enzymes that recognize site-specific DNA targets (Belfort and Roberts 1997). HEGs have almost exclusively been found in Group I introns of fungal mitogenomes (Megarioti and Kouvelis 2020), but they may also be located within Group II introns or as free-standing genes, a characteristic most commonly seen in early-diverging fungal species (Toor and Zimmerly 2002; Megarioti and Kouvelis 2020). In general, HEs may be classified in one of the following four families based on the amino acid motifs participating in the enzyme active site: GIY-YIG, LAGLIDADG, His-Cys box, and HNH (Stoddard 2014). Fungal mt genomes usually contain the first two types; LAGLIDADG is generally the most frequently found and HNH extremely rare (Mukhopadhyay and Hausner 2021; Toor and Zimmerly 2002).

Group II introns rarely carry a LAGLIDADG endonuclease gene, as mentioned above, and they usually include a reverse transcriptase gene that helps splicing and intron's mobility through an intermediate step of reverse transcription (Novikova and Belfort 2017; Mukhopadhyay and Hausner 2021). However, both Group I and II introns can also take part in several intronic arrangements. One of the most complex intron formations involves introns within other introns, a term called twintron (Copertino and Hallick 1991). Twintrons may be formed by different combinations of Group I and II introns, or other intron types (e.g., tRNA introns), and there is no necessity for them to be of the same type (Hafez and Hausner 2015). Twintrons may be formed by different mechanisms which are based on the mobility of their components. The simplest and most common example is when an intron moves to a critical sequence of another one, preventing its splicing until the inside intron moves again and allows for the second one to become splicing competent. There are also cases where the invading intron interrupts an ORF sequence, so the gene is expressed only after its splicing (Guha and Hausner 2014). A characteristic example is the case of the expression of a functional *rps*3 gene located in an external intron, only after the splicing of the internal intron which contains a LAGLIDADG endonuclease in the mitogenome of *Grosmannia piceiperda* (Rudski and Hausner 2012). Similarly, in the fungus *Chaetomium thermophilum*, an ORFless intron interrupts an external intron that harbors an ORF for a homing endonuclease which can only be expressed upon self-splicing of the invading internal intron (Guha and Hausner 2014). In cases where the sequence is not critical for the splicing, the outside intron has the ability to move independently (Hafez et al. 2013). The role and significance of twintron formations is yet unknown, but their complex and various formations suggest that they could be useful as regulatory elements for gene expression regulation through the various alternative splicing mechanisms they provide (Younis et al. 2013; Hafez and Hausner 2015).

In general, the number, as well as length of introns present in mt genomes, is highly variable,

and along with the mitochondrial intergenic regions, introns are major determinants of mt genome size diversity (Hausner 2012; Friedrich et al. 2012; Megarioti and Kouvelis 2020). There are cases, like the fungi *Podospora anserina* and *Phlebia radiata*, where introns account for up to 75% and 80% of the overall size of mtDNA, respectively (Cummings et al. 1990; Salavirta et al. 2014), while in a few other fungal species, i.e., in *Hyaloraphidium curvatum,* no introns were found in their mt genome (Forget et al. 2002). While introns contribute to genome size expansion, intron abundance is not always analogous to fungal mt genome size (Christinaki et al. 2022), and closely related species can vary immensely in the mt genome content of introns. At the same time, intron presence varies among different genes, in respect to both their number and their type. Genes like *cox*1 and *cob* usually contain many more introns than other genes (Freel et al. 2015; Wolters et al. 2015; Christinaki et al. 2022). Accordingly, the various intron types and their HEGs exhibit a preference for certain genes, showing a wide or narrow gene host preference (Hausner et al. 2014; Zardoya 2020; Christinaki et al. 2022).

Introns have a restless evolutionary history. According to the intron-early theory, introns were abundant during the first stages of evolution after the initial endosymbiosis and thereafter had a tendency to disappear. In several cases, an intronic ORF may be fused with a preceding exon in one frame. As suggested in the modified Goddard and Burt model, mutations, which change the target sites for intron acquisition and alternative splicing, may lead to the loss of the intronic ORF fused to the upstream exon (Goddard and Burt 1999; Guha et al. 2018). Recent studies have shown, however, that introns may remain intact at conserved gene locations (Korovesi et al. 2018; Wai et al. 2019), a fact that indicates a form of habituation in certain genes. In reality, introns not only stay intact without disappearing, at least as frequently as the intron-early theory suggests, but may in some instances become more numerous, with new introns being acquired through HGT events or transposition (Wu et al. 2015). Introns and HEGs have a long and co-related evolutionary history. According to the recently proposed "aenaon" model, introns have undergone a complex evolutionary transformation, from an ancestral compact form to a more expanded and complex structure. HEGs tend to invade introns throughout evolution in a perpetual manner, through many recombination, transposition, and horizontal gene transfer events. Ancestral introns still exist, while new introns may be found in Dikarya fungi (Megarioti and Kouvelis 2020). Finally, it has been shown that intron diversity and mobility within the genome play a substantial role in recombination events. Introns tend to move from an intron-containing allele to an intron-minus allele in a homology-dependent gene conversion, a term called intron homing (Dujon 1989). During this process of intron movement, sequences from the intron-containing allele are transferred to the recipient gene, thus shaping genetic diversity through intron homing (Repar and Warnecke 2017; Wu and Hao 2019).

3.10 Unidentified ORFs with Other Function than Intron Splicing and Mobility

In addition to the conserved fungal mt genes or Group I and II intronic ORFs that have an already known role (i.e., HEGs, RT and *rps*3), mitochondria also contain a number of non-conserved ORFs (ncORFs), otherwise called unidentified ORFs. Their presence varies substantially among fungal mt genomes and can either be found in introns or as free-standing genes within intergenic regions (Liu et al. 2020b; Losada et al. 2014; Duò et al. 2012). There have been three main theories regarding their origin: (a) they are derived from existing mt loci that have accumulated point mutations, (b) they are derived from a nuclear gene that was transferred in the mitochondrion, and/or (c) horizontal transfer has occurred from another organism. In addition, mobile elements, such as HEGs, can alter ORFs so they could possibly create unidentified ORFs (Al-Reedy et al. 2012).

Despite being numerous, the majority of them have unknown roles (Hane et al. 2007; Duò et al. 2012). Regardless, it has been suggested that a large number of nuclear ORFs are typically found in large fungal mt genomes, and thus, they may contribute to genome size expansion (Li et al. 2015). Mitochondrial genomes of *Fusarium* spp. were found to contain highly variable regions of 7–9 kb that encode Group I intron associated ORFs, as well as an exceptionally large unidentified ORF which was probably horizontally transmitted before the divergence of *Fusarium* spp. (Al-Reedy et al. 2012). Similarly, free-standing unidentified ORFs were also located in the mt genome of four *Rhynchosporium* spp., among which there was a large unidentified ORF, i.e., Ro_ORF11, which encoded an 807 amino acid in size protein with five transmembrane domains without a recognized function (Torriani et al. 2014). It has also been suggested that non-conserved ORFs with unknown functions may be involved in mitonuclear interactions (Clergeot and Olson 2021). Such interactions can contribute to virulence and host interactions of pathogenic fungi (Hu et al. 2020).

Unidentified ORFs' random and non-conserved presence in fungal mt genomes, in combination with their location within introns or intergenic regions, implies that they contribute largely to mt diversity among different species/taxa. This is in compliance with their role as a rapidly evolving accessory compartment in the mt genome, as their location in non-conserved areas allows them to be the subject of mutations or other genetic rearrangements (Croll and McDonald 2012; Torriani et al. 2014).

3.11 Genetic Codes

The canonical genetic code was established before the LECA. Its essential role in protein synthesis suggested that its alteration is not promoted or favored, as such a thing would cause an alteration in the amino acid sequences and thus possibly in protein structure or function (Crick 1968). As more genome sequences become available, it is now clear that there are numerous deviations throughout different groups of organisms, and indications exist that there is a greater evolvability and flexibility of the code (Knight et al. 2001a, b, c). However, this suggests that the various established genetic codes would require deep structural, functional, and evolutionary changes, which are deep modifications. Still, these have not yet been elucidated upon. Mitochondrial genomes employ variable non-standard genetic codes compared to their nuclear counterparts (Swire et al. 2005). Fungal mt genomes, in particular, employ the genetic codes NCBI 4 (The Mold, Protozoan, and Coelenterate Mitochondrial Code and the Mycoplasma/Spiroplasma Code) and NCBI 3 (The Yeast Mitochondrial Code), while only a few of them employ genetic code NCBI 1 (The Standard Code) and NCBI 16 (Chlorophycean Mitochondrial Code) (Fonseca et al. 2021). Deviations from the canonical code involve reassignments between different amino acids or from amino acids to stop codons and vice versa. Genetic code 4 (UGA codon from Stop to Trp) is the most common change used in fungal mitochondria, while genetic code 3 is mostly used by yeast species (AUA from Ile to Met, CUN from Leu to Thr, and UGA from Stop to Trp). Genetic code 1 is found in species of Mucoromycota and *Schizosaccharomyces pombe* and genetic code NCBI 16 (UAG from stop to Leu) in Chytridiomycota (Laforest et al. 1997).

Sengupta et al. (2007) summarize the reassignments that occurred after the establishment of the canonical code and classify them according to a gain-loss framework. In brief, "loss" is the deletion of the gene for the tRNA originally associated with the codon to be reassigned or the loss of function of this gene due to a mutation or base modification in the anticodon. "Gain" is the addition of a new type of tRNA for the reassigned codon through gene duplication, or the gain of function of an existing tRNA due to a mutation or a base modification, enabling it to pair with the reassigned codon. Therefore, four possible mechanisms for codon reassignments (Sengupta and Higgs 2005; Sengupta et al. 2007) were proposed (Fig. 3.2):

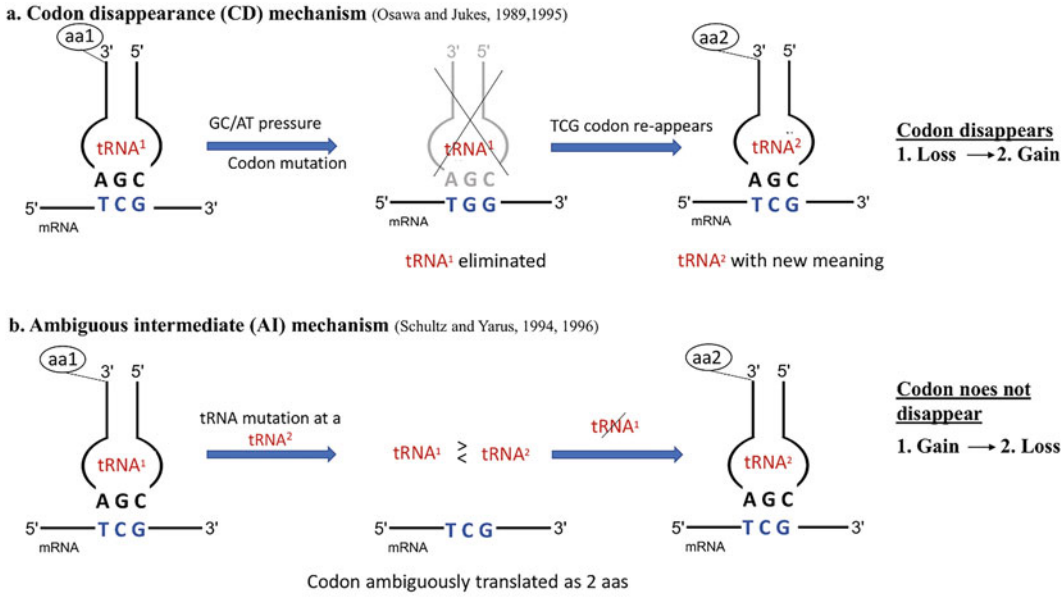

Fig. 3.2 Schematic representation of the two most widely accepted models for codon reassignments

codon disappearance (CD), unassigned codon (UC), ambiguous intermediate (AI), and compensatory change mechanism. CD involves codon loss prior to any other gain and loss events, a disappearance occurring through random drift and directional mutation pressure. It has been previously suggested that variation in GC content is a major determinant in the CD mechanism and influences synonymous codon usage (Knight et al. 2001b). This hypothesis, alternatively stated as the "codon capture" model by Osawa and Jukes, suggested that this mechanism would minimize the possible catastrophic effects of codon alteration before its disappearance (Osawa 1995; Osawa et al. 1992). In both the unassigned codon (UC) and ambiguous intermediate (AI) mechanisms, codons do not disappear prior to the reassignment, but *trn* loss and gain occur first, respectively (Schultz and Yarus 1996). None of the above models can fully interpret all genetic code alterations, and most importantly, they are not mutually exclusive. Sengupta et al. (2007) suggest that CD mechanism may explain stop codons to protein codon changes, such as UGA from stop to Trp (genetic code 3) or UAG to Leu (genetic code 16), as well as some of the other reassignments, because stop codons are rarely found in the mt genome, and thus, are more likely to be lost by chance. In addition, CD mechanism may explain the reassignment of CUN and CGN codons in some yeast species, as it appears that there is a strong bias in their mt genome against C, which leads to codon loss. In contrast, Knight et al. (2001b, c) support the AI mechanism, which acts alone or after the codon disappearance.

All models described above may be used to explain differential appearance of their genetic codes in fungal mitogenomes, but it is important to have in mind that changes may have happened independently many times throughout evolution. In favor of this argument is gene codon usage employed in mitogenomes of early-diverging fungal species and other species from Basidiomycetes and Ascomycetes. Specifically, recently reassigned codons like UGA (a stop codon in the universal genetic code) have only recently been utilized, in mt genomes in Ascomycetes, and randomly in the respective genomes of Basidiomycetes, while they are absent in the respective genomes of early-diverging fungal fungi (Korovesi et al. 2018).

Similarly, the occurrence of alternative codon usage in the mitogenomes of yeasts is variable even within members of the same families (Christinaki et al. 2022), thus supporting the incidental but restless change of genetic codes throughout evolution.

3.12 Major Fungal Evolutionary Transitions Based on Mitogenomes (Conclusions)

The analyses of all the features of fungal mitogenomes, as summarized above, offer many insights pertaining to the evolution of eukaryotes within the kingdom of fungi. Likewise, they provide insights into the evolution of the alpha-proteobacterial endosymbiont which was "adopted" by the archaeon acting as the proto-eukaryote, according to the prevailing endosymbiotic theory (Martin et al. 2015).

From the above-mentioned analyses, it becomes evident that a diverse group of species, like the one belonging to the kingdom of fungi, carries all the conserved ancestral elements, such as genes for the subunits of oxidative phosphorylation, since they cannot be transferred to the nucleus (Adams and Palmer 2003; Daley and Whelan 2005). At the same time, these ancestral elements are differentiated and evolved with the addition of mobile elements like introns and HEGs (Megarioti and Kouvelis 2020; Mukhopadhyay and Hausner 2021), plasmids, and DNA/RNA polymerases (Formighieri et al. 2008; Fricova et al. 2010). There is a restless "back and forth" mechanism in mitochondrial genomes, which is based on recombination and horizontal gene transfer (HGT) events. Even though this is not a deterministic procedure, but a rather stochastic one, it presumably helps the species carrying these genomes to better adapt to their environment. Under this context, studies like the one of Clergeot and Olson (2021) provide evidence that there is a correlation of mt ORFs with pathogenicity, due to their interaction with their nuclear counterparts. NUMTs have revealed an ongoing mechanism of ceaseless interaction between nc chromosomes and mt genomes (Sacerdot et al. 2008; Beaudet et al. 2014), thus offering fungal organisms the ability to evolve with the creation of new genes and pseudogenes or gene hitchhiking (Noutsos et al. 2007; Xiao et al. 2017). Moreover, mt plasmids are, beyond any doubt, involved in processes like senescence and life-span determination (Hermanns et al. 1994; Monteiro-Vitorello et al. 2000) and along with introns, tRNA genes and repetitive sequences may act as hotspots for recombination within the genome and eventually lead to gene shuffling, under a model similar to the one already proposed for the mobility of Group I introns and their HEGs, i.e., the "aenaon" model (Megarioti and Kouvelis 2020). In other words, all mitogenomic studies which tried to decipher the evolution of these genomes and the fungi carrying them revealed that all known mechanisms needed for evolution, including genetic drift, genetic draft (gene hitchhiking), and HGT, also apply in the fungal mitogenomes (Lang et al. 2014; Xiao et al. 2017; Hao 2022; Christinaki et al. 2022). Moreover, unique (to our knowledge) mechanisms in fungal mitochondria, such as the programmed translational jumping in mt genomes of yeasts (Lang et al. 2014), provide evidence that evolution may happen indiscriminately at any time, adding to the plasticity and diversity of mitogenomes. Finally, mitogenomes and studies which try to examine nucleus–mitochondrion interactions in the context of the mitogenome's well-tempered functioning, such as the coevolution of the major proteins implicated in the transcription of mt genes, help in further supporting the proposed hypotheses of mt genome changes through different genetic mechanisms (Varassas and Kouvelis 2022). As mentioned earlier, recent analyses examining fungal mt transcription led to support for the hypothesis that the bacterial endosymbiont was most probably infected with a T7-like phage, and this prophage genome offered the RNA polymerase to the newly formed mitochondrion (Filée and Forterre 2005; Varassas and Kouvelis 2022). Needless to say, other fungal studies, which cannot be overruled, suggest that this phage RNA polymerase, as well as the DNA polymerase, both presenting a phage origin of the mt respective

enzymes, might have been acquired in the mitochondrion at a later stage (Burger and Lang 2003).

Irrelevant to which theories are the correct ones, it is evident that the fungal mitogenome diversity offers data needed to resolve such crucial questions such as the origin of the mitochondria and how mitogenomes evolve over time. Therefore, in this era of broad sequencing and WGS projects, the mitogenome analyses of fungal species must be performed in depth.

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