EXTRACHROMOSOMAL GENETIC ELEMENTS IN FUNGI

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All fungi like eukaryotes possess mitochondria, which are the sites of the oxydative phosphorylation. As eukaryote evolution depends on oxygenic atmosphere, these organisms are primarily aerobic. Except a small group of strict anaerobes (those which lost the capacity of oxydative pathways living in special milieu in association with rumen of grass-eating animals) all fungi can utilize various compounds as carbon sources via oxidative phosphorylation pathways resulting in high energy yield. Certain groups of fungi – i.e. most of the yeasts – under anaerobe conditions, are able to supply themselves with lower levels of fermentation energy, too exhibiting a slow growing capacity utilizing the same amount of carbon source. The mutation of mitochondrial genome or mitochondrial functions encoded by nuclear genes of these fungi might result in a so-called *petite* phenotype producing small colonies on solid media due to their slow growing capacity. These mutants can utilize only fermentable carbon sources. Filamentous fungi have only limited possibilities to produce such phenotypes. Except *Zygomycetes* (where the shortage of oxygen induces dimorphic transitions) filamentous fungi can grow and develop their vegetative and sexual reproductive structures only in aerobe milieu. However among *Neurospora* species there are several mitochondrial mutations resulting in morphological phenotypes. These are due to the lower energy level provided by the reduced capacity of cytochrome-oxidase enzymes. These mutants (e.g. *poky*, *stopper*) can be considered as *petite* analogues. The complete loss of mitochondrial functions – such as *rho zero* character in yeast – cannot be survived by filamentous fungi. *Podospora anserina* and some of its close relatives exhibit a so-called *senescence* phenotype, which means that the growing hyphae in the youngest part of the colonies stop growing and start to die within a short period of time. This phenomenon – discussed below – is also connected to reduced function of mitochondria.

The first part of this paper gives a short overview of the genetic organization of mitochondria of fungi, based on the most recent data of three filamentous fungi: *Aspergillus nidulans*, *Neurospora crassa* and *Podospora anserina*. Their data are compared to those of the well-characterized *Saccharomyces cerevisiae*. In the second part we summarize what we know about other extrachrosomal elements, such as DNA

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plasmids of various origins and structures, and dsRNAs or virus like particles (VLP). Also discussed are their roles and/or putative functions in the life of the fungi.

Mitochondrial DNA

The fungal mitochondrial genomes vary in size between 17.6 and 172 kb represented by *Schizosaccharomyces pombe* and *Agaricus bitorquis*, respectively [1, 2]. This range puts the fungi in the middle in respect of sizes of mtDNAs between animals and plants. Animals (including humans) exhibit the smallest size of mtDNAs between 14–36 kb (but most of them range in size between 16–18 kb). These very compact genomes proved to be intronless, and size differences can be due to the variability of their replicating (*ori*) sequences. Plants possess the largest mtDNAs (from 218 to 2400 kb). Despite their large sizes they have no additional functioning genes and their intron content is abundant resulting in very complex RNA editing [1, 3].

MtDNA organisation in fungi has been reviewed by several authors [1–11]. Despite some organisational differences among various groups of fungi several generalisations can be made. The configuration of mtDNAs is considered to be double stranded circular, but recently there have been some doubts about this general statement. Based on various molecular techniques verifying the structure of mtDNAs they are now considered to have linear variations especially during their replication [12].

The most common mitochondrial functions encoded by mitochondrial sequences are as follows: 1) A part of the translation apparatus with minimum set of 22 tRNAs and ribosomal RNAs: *rns* and *rnl*, small and large subunits of rRNAs, respectively; one protein of the small ribosomal subunit (*var1p*); a 9S RNA component of mitochondrial RNase in *S. cerevisiae*; 2) membrane associated proteins responsible for catalyzing oxidative phosphorylation: seven subunits of NADH dehydrogenase complex (*ndh*) (except *S. cerevisiae* and most of the yeasts); apocytochrome b (*cob*) (cytochrome b, c1 complex); three subunits of cytochrome c oxidase (*cox*) and ATP synthase subunits 6, 8 and 9 (*atp*) [1, 4, 6, 8, 11]. Additionally URFs are present with unknown functions.

As only a limited number of mitochondrial proteins (about 8–10% of them) are encoded by mitochondrial genes it follows that all the others related to mitochondrial functions and structural elements have to be encoded by the nucleus. Distribution of functioning genes of ATP-ase subunits is a good example for co-operation between mitochondria and nuclei. *Podospora anserina* has no mitochondrially encoded *atp9* gene. Both in *S. cerevisiae* and *S. pombe* the *atp9* subunits are active in mitochondria, but there are no such functioning nuclear genes of these yeasts [1, 11]. *A. nidulans* possess *atp9* genes both in nuclei and mitochondria. It seems that the mitochondrial *atp9* gene proceeds transcription, but translational product is not produced especially during the vegetative growth similar to *N. crassa* [4, 6]. Some of the nuclear encoded proteins can enter mitochondria, across the specific double membrane boundaries. Studies on these processes e.g. the role and functions of mitochondrial heat shock proteins, chaperons have become one of the most exciting fields of research in modern cell biology [13].

Mitochondrial introns make mosaic structure of the functioning genes, they have self-splicing activity and usually contain open reading frames (ORF). Two main groups of introns were distinguished on the basis of self-splicing excision processes. These group I and group II introns are characterized by specific secondary structures which strongly determine their splicing processes. The introns generally carry ORFs with endonuclease, or reverse transcriptase activity in the cases of group I and group II introns, respectively. There ORFs are responsible for mRNA maturing processes (maturase activity) and the mobility of whole introns or only their ORFs [14]. Of course a great part of the splicing of mitochondrial primary transcripts *in vivo* is dependent on nuclear encoded maturases. While group I introns encode endonucleases, *rnl* intron's ORF of *N. crassa* encode a ribosomal protein (S5).

Except *Zygomycetes*, most of the fungi, possess mtDNAs with exceptionally high AT-rich sequences when compared with the nuclear ones (this provides an easy separation of them by density-gradient ultra centrifugation) [15]. Among the ascomycetous yeasts *S. cerevisiae* possesses relatively large mtDNA (75 kb) but only about one-fifth of the total mt genome represents exon sequences. The size of mtDNAs can be influenced by the number of introns in specific genes in addition to the intergenic spacer regions [9–11]. In the long non-coding spacers (intergenic sequences) of *S. cerevisiae* the AT distribution ranges 85% but even coding regions can contain about 75% AT sequences. However, the total GC content is only about 18%; large numbers of short GC clusters could be identified in intergenic spacers and in some functioning regions of it, such as *ori/rep* sequences (their numbers are at least 8, scattered around the whole genom). GC clusters can be classified into different groups, can form short duplications and direct repeats and have function in intramolecular recombinations and excision of *petite* mtDNA.

The mtDNAs of filamentous *Ascomycetes* are also AT-rich as compared with coding regions. The intergenic regions are highly variable, their sequences are species specific. In the mt genomes of well-studied *N. crassa* and *P. anserina* dispersed repeated elements have been detected. These short sequences with high GC content are present in several dozen copies: e.g. *Pst*I palindromes in *Neurospora* [1, 6], MUSEs elements in *P. anserina* [20]. These special motifs are thought to be involved in intramolecular processes of mitochondrial sequences.

Comparing mitochondrial functions and gene content with the sizes of mtDNAs in several species some generalizations can be made.

Aspergillus nidulans has mtDNA with the size of 31.5 kb, nearly the full sequence is known. Its tRNAs are located in two big clusters except seven of the total 28 tRNA genes. All seven *ndh* subunits are present, beside cob and three *cox* subunits (*cox1, cox2, cox3*), all the three *atp* subunits (*atp6, atp8, atp9*) are also present in mtDNA. A nuclear gene encodes the *atp9* function, too. *A. nidulans* possess five group I introns, their locations are as follows: one-one is in cob and *rnl* regions and three are situated in *cox1* subunit, together they form a mosaic gene structure. Mitochondrial drug resistant mutants are known such as oligomycin-, chloramphenicol-, mucidin resistance and cold-sensitivity [4]. MtDNA polymorphisms have not been reported even between vegetative incompatible *A. nidulans* isolates. Only its closely related species (*A. quadrilineatus* and *A. echinulatus*) exhibited slight intronal variations [16]. Black *Aspergilli* (sectio *Nigri*) exhibit a wide range of mtDNA polymorphism [17–19]. Beside the existing great intra-

and interspecific variations of RFLPs the sizes of the mtDNAs also differ greatly due to the actual intron content.

Neurospora crassa laboratory strain (Oak Ridge 74-OR-23-1A) bears 62 kb mtDNA. Its 27 tRNAs are clustered similarly to *A. nidulans*. The gene content in other respects is also similar to it, but *N. crassa* possess five URFs (*A. nidulans* has one URF: unidentified reading frame) and 9 introns, too. The *cox1* region is intronless, *cob* and *ndh5* genes have two introns, one can be detected in each of the *ndh1*, *ndh4*, *ndh4l* and *atp6* subunits and every one of this intron bears a single ORF. The intron situated in *ndh3* subunit has two ORFs due to frame shift transcription. All introns of laboratory strains are proved to be group I types encoding endonucleases as maturase functions. Similarly to *A. nidulans* small numbers of promoter sequences (five) have been identified, this resulted in a limited number of long pre-mRNAs. This finding is contrary to those of the yeasts where more promoters can be recognised. No drug resistant mutants could be isolated, but some morphological mutants could be produced. The phenotypes of *poky* or *stopper* mutant strains are due to the reduced level of oxydative phosphorylation. Ethidium-bromide, acriflavine, nitroso-guanidine derivatives can induce such mutant phenotypes, deleting specific parts of mitochondrially encoded enzyme genes resulting in various levels of deficiency in cytochrome *aa3* and b, but cytochrome c is usually not effected by these treatments. All three types of morphological mutants can be identified. Two of them cannot be suppressed by nuclear gene functions, in these cases the energy requirements are generated by the cyanide insensitive alternative respiration system. Among wild isolates of *Neurospora* strains considerable mtDNA polymorphisms have been observed [6, 7]. In a wild-type strain a group II type intron with reverse transcriptase activity has been detected in *cox1* gene [21].

Podospora anserina: mtDNAs vary in size between 80–102 kb. Well-studied "s" and "A" heterokaryon incompatible strains bear 94.192 and 100.314 bp, respectively. Nearly all of the *mt* genomes are sequenced. The laboratory strains possess 33 group I type and 3 group II type introns. About 60 kb from the total 95 kb mtDNA are covered by intron sequences. In particular the *cox1* gene is interrupted with at least 16 introns, therefore this mosaic gene itself represents 22.9 kb size. *P. anserina* mitochondrial DNA codes for 25 tRNA genes, but has no *atp9* subunit [8]. So-called mitochondrial ultra-short invasive elements (MUSE) have been detected (GGCGCAAGCTC), thought to be the sites of intramolecular recombinations [20]. Among the wild isolates the mtDNA polymorphism are exceptionally rich due to their variable intron contents [22].

Senescence phenomenon (progressive loss of growth potential ends in death) has been extensively studied in *P. anserina* where this is a normal event in wild type strains [23–26]. Senescence is correlated with the appearance of circular multimeric plasmid like elements in mitochondria. Senescence DNAs (senDNA) are amplificates of specific mobile intron sequences. When the introns are excised they are called plasmid-like DNA. There are several types of senDNAs, α, β are the most common ones. The α-senDNA derives from the first intron of cox1 gene by the precise excision of it, therefore it always has the same monomeric size around 2.5 kb. This intron can be grouped with class II introns, which have ORFs coding reverse transcriptase-like proteins. The size of putative α-protein is about 96–100 kD. Various natural senescence cultures may exhibit different α-introns, but all of them proved to be reverse transcriptase homologues. Following

excision the amplified α-introns can rearrange the mitochondrial genome. As only about 30 kb of the total mtDNA remains in original structure, this rearrangement could result in reduced activity of cytochrome a3 and b proteins. Excised β-senDNAs are larger than the α-introns, they differ in length but share a common sequence of 1.1 kb which is located between the 3' end of *cox1* and *nadh4l* genes. β-senDNAs can be classified as group I introns. Their common region has three ORFs X, Y and Z, the last encodes for a glycine-rich ssDNA-binding protein. The reason of senescence is the specific structure of the mtDNA itself, but the occurrence of this phenomenon depends on many other factors. There is a special nuclear control, e.g. in *grisea* and *vivax* double morphological mutants the senescence phenotype has never been observed, and could not occur in liquid phase. The amplified sequences of mobile introns result in a decreased energy level, by the rearrangement of functioning *mt* genome that induces a complex ageing process. This process can be suspended by ethidium-bromide treatment, probably by the selective inhibition of the excised intronal DNAs. Some natural occurring "rejuvenation" events have also been observed, e.g. in presence of a linear mitochondrial plasmid (pAL2-1) the senescence cultures turn into juvenile ones.

In *Neurospora* besides the *poky* and *stopper* mutant phenotypes the real senescence phenomenon can be studied in wild type plasmid bearing strains [27–29]. One-third of natural isolates of *N. intermedia* from the Hawaiian island of Kauai exhibit senescence caused by the so-called *kalilo* plasmid (or *kal*DNA) its size is about 9 kb. This is a linear protein primed replicating plasmid that has no homologous region with *mt-* or *nuc*DNAs. It has two ORFs encoding for DNA and RNA polymerases, respectively. This element could exist either in a free form (AR-*kal*DNA) or integrated into various sites of the *mt* genome (IS-*kal*DNA), most frequently into the LrRNA gene. In contrast to *Podospora* the amplified free plasmids integrate into the intact *mt*DNAs. The senescence can be observed only in the presence of this plasmid, but the rearrangement of mtDNAs itself caused by the integration does not result in death. Some nuclear controls are also involved in this process. Another linear mitochondrial plasmid is the so-called *maranchar* derived from an Indian isolate of *N. crassa*. Although *mar*DNA and *kal*DNA do not show homology to each other, they have the same basic structure and use the same mechanism for replication. Both plasmids have terminal inverted repeats (TIR) which play a role in the integration. In contrast to *kalilo* and *maranhar* of *Neurospora*, linear plasmids of other filamentous fungi are not associated with senescence.

In *Aspergillus amstelodami* the so-called *ragged* (*rgd*) mutant exhibits a senescence-like phenotype. Various regions of mtDNAs are excised and amplified and form head-tail repeated circular concatamers. In the presence of these molecules the level of oxydative phosphorylation decreases. In contrast to *Podospora* the *A. amstelodami rgd* strains contain intact mitochondria, too [30].

Fungal plasmids

Senescence, as it is demonstrated above, involves the presence of plasmids or plasmid-like elements as liberated mobile introns. These plasmids belong to the group of

mitochondrial plasmids because of their location. They are called defective plasmids or defective sequences owing to causing progressive loss of growth. Most of the fungal plasmids are neutral they do not result in any phenotypical changes in the host strains. In contrast to the mostly circular dsDNA plasmids of bacteria the fungal ones are quite different resulting in various phenotypic consequences. They differ in their basic structures, types of nucleic acids, their origin and location, and finally in their functions (they could be cryptic or responsible for certain phenotypes) [27, 28, 31, 32].

Plasmids of mitochondrial origin occur most frequently among filamentous fungi. These plasmids can be classified into the following groups:

1) mtDNA origin defective sequences: as sen-plasmids in *Podospora*, kalilomaranhar-plasmids in *Neurospora*.

2) so-called true mitochondrial plasmids having no homology to *mt*- or *nuc*DNA. This group involves circular and linear plasmids.

Well-known circular plasmids were described from various natural isolates of *Neurospora*. Among them *Mauricewille* and *Varkud* were studied in detail, they originated from *N. crassa* and *N. intermedia* with nearly the same size of 3.6 and 3.7 kb in monomeric conditions, respectively. They exhibit a high level homology, having nearly common ORFs encoding a reverse transcriptase, which is a typical class II intron characteristic. These plasmids replicate thorough mRNA intermediates, they also bear class I type intron-like sequences, and *Pst*I palindromes which are characteristic in *mt*DNA, too. Various origin of *N. tetrasperma* strains possess other homologous group of circular plasmids e.g. *surinam, hanelei*, while *fiji* plasmid from *N. intermedia* also belongs to this group with their sizes of around 5 kb. *LeBelle* plasmid also derived from a *N. intermedia* strain from another geographic location representing another homologous group. This is only one circular *mt* plasmid, which shares homologous regions with the *mt*DNA. The fourth homologous group of *Neurospora* circular elements the so-called VS-plasmids (*varkud satellit*) have relatively small sizes varying between 1.8 and 2.6 kb. VS-RNAs are transcripts of VS-plasmid exhibiting a self-splicing activity *in vitro*. The presence of these plasmids cannot be correlated directly with any phenotypes, but as mobile retroelements are able to induce rearrangement of genomes resulting in genetic instability during the vegetative growth. Some other practically important fungi as *Cochliobolus heterostrophus*, *Trichoderma viridae*, *Ophiostoma novo-ulmi* also possess circular mitochondrial plasmids. Except the last one no phenotypic character appears to be present in these elements.

Linear mitochondrial plasmids of fungi have a common structure called: protein primed replicating elements. They have terminal protein (TP) at the 5' end of the genome and terminal inverted repeats (TIR) with different sizes. The best studied of this type are *kalilo* and *maranhar* resulting in senescence, but many others are cryptic. *Claviceps purpurea*, *Ascobolus immersus*, *Ascosphera apis*, various *Fusarium* strains and basidiomycetous *Lentinula edodes*, *Agaricus bitorquis*, *Pleurotus ostreatus*, *Rhizoctonia solani* contain linear protein primed plasmids without real phenotypic consequences. Their sizes vary between 1.1 and 10 kb. In presence of plasmids some *Fusarium* strains do not produce trichotecin type mycotoxins probably due to the decreased energy level caused by plasmids resulting in inability of epoxidation step that would be required during mycotoxin synthesis. The pAL2-1 plasmid of *P. anserina*, which results in rejuvenation in

senescence cultures, also belongs to this class of plasmids [32]. True slime mould *Physarum polycephalum* has mitochondrial linear plasmids with an essential function. These plasmids direct fusion of mitochondria during sexual mating and also direct the mtDNA recombination between parental strains which is a unique characteristic among the lower eukaryotes [33].

Linear protein primed replicating plasmids can also be localized out of mitochondria either in cytoplasm or in nuclei. The best example for cytoplasmatically localized linear plasmids are *Kluyveromyces lactis pGKL1* (8.9 kb) p*GKL2* (13.4 kb) which are responsible for killer phenomenon in this species. Details are presented in an other paper [34].

The well-studied 2 μ plasmid of yeast represents a completely different type of such extrachromosomal elements, they are circular and located in the nucleus. Until now it is the best eukaryotic vector for transformation, its ARS elements are frequently used in shuttle constructions. Its completely sequenced gene's functions are known, but their role in host cells has not yet been elucidated.

dsRNAs and VLPs in fungi

Most of the fungal viruses are dsRNAs containing VLPs. The majority of them have no influence on the fitness of the fungal growth (in contrast to the first discovered fungal virus causing so-called "*la France disease*" symptoms in *Agaricus bisporus*). They are so-called endogenous, non-pathogenic viruses, their presence do not result in any advantage or disadvantage for host cells. They can be transmitted under experimental conditions, in nature they spread via cytoplasm fusion and are inherited maternally in general. Taxonomically the dsRNA fungal viruses can be grouped into three families. The best-studied members of *Totiviridae* is the so-called killer virus (or RNA killer plasmids) of *Saccharomyces cerevisiae* [35]. It consists of two linear dsRNAs, one of which is responsible for toxin production and carries a gene for immunity (MdsRNA). The other RNA (L-dsRNA) is a 4579 bp molecule with two long ORFs. ORF1 encodes for a 76 kD viral particle major coat protein. ORF2 occupies the 3' part of the L-A positive strand and overlaps ORF1 in –1 frame by 130 bases, and apparently encodes for an RNA-dependent RNA-polymerase, similar to that of (+)ssRNA viruses. Similar RNA bearing killer phenomena were described in connection with some other ascomycetous and basidiomycetous fungi e.g. *Ustilago maydis*, and *Cryptococcus* species. The members of the *Partitiviridae* family became well known as viruses of the *Penicillium chrysogenum*-group. Some other ascomycetous fungi also possess cryptic dsRNA viruses with segmented genomes e.g. *Gaeumannomyces graminis* and many *Aspergillus* and *Penicillium* species. The functions of these viruses are not known but there are some hypotheses that they might influence the gene expression of host as effector molecules responsible for silencing and co-suppression [36]. *Cryphonectria parasitica*'s hypovirus is the most studied member of the *Hypoviridae* family where the viruses are not always encapsidated. This fungus is a causative agent of chestnut blight and has killed millions of chestnut trees in America. Natural virus transmission is inhibited by vegetative

incompatibility, therefore its possible application as a biocontrol agent has not yet been successful. The molecular background of hypovirulence has been extensively studied. Virus free *C. parasitica* transformed by cDNA of hypovirus can induce phenotypic changes characteristic to hypovirulence strains. Hypovirulence is attributed as a loss of aggressive extracellular enzyme activities of the pathogen fungus [37].

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