Evolution of eukaryotic introns following endosymbiotic gene transfer

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Für meine Familie

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1 Abstract

Spliceosomal introns are segments of non-coding sequences in eukaryotic genes. After transcription of a gene, introns are excised from the pre-mRNA by the spliceosome before translation into a functional protein. Spliceosomal introns and the spliceosomal machinery have been identified in all eukaryotic genomes and are absent from all prokaryotic genomes sequenced to date. The origin of spliceosomal introns is mainly discussed in the context of two different hypotheses. The introns-early hypothesis states that spliceosomal introns were present in the last common ancestor of prokaryotes and eukaryotes but were subsequently lost in all prokaryotes. In contrast, the introns-late hypothesis links the origin of spliceosomal introns to the emergence of eukaryotes.

Similarities between splicing mechanisms of spliceosomal introns and those of group II introns support the opinion that they might have had a common ancestor. The fact that group II introns are present in bacterial and mitochondrial genomes, leads to a possible evolutionary connection between spliceosomal introns and mitochondria. These cell organelles originated from endosymbiosis with an α-proteobacterial ancestor in a host cell. During evolution, the mitochondrial genome was reduced and many genes were lost or transferred to the host genome, a process known as endosymbiotic gene transfer. This process might have spread group II introns into the host genome, and subsequently might have initiated the evolution of spliceosomal introns and the spliceosome. These influences could also have created a selective force towards the evolution of the nucleus which separated splicing from translation.

Nuclear genes that originated in endosymbiotic gene transfer did not contain spliceosomal introns when they were transferred to the host genome, so that introns in these genes were all gained after integration into the genome. To gain insight into the evolution of spliceosomal introns, the intron/exon structure of nuclear encoded proto-mitochondrial genes of the oxidative phosphorylation pathway and genes of mitochondrial ribosomal proteins were examined. Homologs of 64 human proto-mitochondrial proteins were identified in 18 eukary-

otic species. The timing of gene transfer events within the species phylogeny revealed endosymbiotic gene transfer as a very dynamic evolutionary process in general.

A database annotation independent method was developed to identify intron positions using the sequence information. The analyzed characteristic features of spliceosomal introns in the genes under consideration revealed a highly dynamic and species specific intron evolution. Intron densities and phase distributions as well as a predominance of shared intron positions between animals and plants are in accordance to other results found in eukaryotic genes or genomes. No correlation was detected between the time of the transfer and the intron density, which suggests a high rate of intron gain after the integration of the genes in the host genome.

A clear case of parallel intron gain in animals and a green alga was found in the gene *nad7* which was transferred independently in these species. This rare example supports the opinion that shared intron positions are not implicitly conserved.

2 Zusammenfassung

Spleißosomale Introns sind nicht-kodierende Sequenzabschnitte in Genen von Eukaryoten. Nach der Transkription eines Gens werden die Introns mit Hilfe des Spleißosoms aus der prä-mRNA herausgeschnitten, bevor das Gen in ein funktionelles Protein translatiert wird. Spleißosomale Introns und der Spleißosomenkomplex wurden in allen bisher sequenzierten eukaryotischen Genomen identifiziert, in prokaryotischen Genomen sind sie jedoch nicht vorhanden. Der Ursprung spleißosomaler Introns wird hauptsächlich im Kontext von zwei unterschiedlichen Hypothesen diskutiert. Die "introns-early" Hypothese gibt an, daß spleißosomale Introns in dem frühesten gemeinsamen Vorfahren der Prokaryoten und Eukaryoten vorhanden waren, die anschließend in allen Prokaryoten verloren gingen. Die "introns-late" Hypothese hingegen verbindet den Ursprung der spleißosomalen Introns mit der Entstehung der Eukaryoten.

Ähnlichkeiten zwischen den Spleiß-Mechanismen von spleißosomalen Introns und denen der selbstspleißenden Gruppe-II Introns unterstützen die Annahme, das sie einen gemeinsamen Vorfahren besitzen. Die Tatsache, daß die Gruppe-II Introns in den Genomen von Bakterien und Mitochondrien vorkommen, führt zu einer möglichen evolutionären Verbindung zwischen spleißosomalen Introns und Mitochondrien. Diese Zellorganellen entstammen einer Endosymbiose eines α-proteobakteriellen Vorfahren in einer Wirtszelle. Im Laufe der Evolution wurde das mitochondriale Genom reduziert indem viele Gene verloren gingen oder zu dem Wirtsgenom transferiert, der Prozess des endosymbiontischen Gentransfers. Dieser Prozess könnte die Verbreitung der Gruppe-II Introns im Wirtsgenom verursacht haben und anschließend die Evolution der spleißosomalen Introns und des Spleißosoms eingeleitet haben. Diese Einflüsse könnten auch eine selektive Kraft zur Begünstigung der Bildung eines Zellkerns hervorgerufen haben, welcher das Spleißen von der Translation trennt.

Zellkernkodierte Gene die dem endosymbiontischen Gentransfer entstammen, enthielten keine spleißosomalen Introns als sie in das Wirtsgenom transferiert wurden, so daß diese Introns erst nach der Integration im Wirtsgenom

eingefügt wurden. Um Einsicht in die Evolution von spleißosomalen Introns zu gewinnen, wurden die Intron/Exon Stukturen von zellkernkodierten proto-mitochondrialen Genen des Oxidativen Phosphorylierungsstoffwechselwegs und von Genen mitochondrialer ribosomaler Proteine untersucht. Ausgehend von 64 proto-mitochondrialen Proteinen des Menschen wurden Homologe in 18 eukaryotischen Spezies identifiziert. Die zeitliche Einordnung der Gentransferereignisse innerhalb der Phylogenie der Spezies zeigte den endosymbiontischen Gentransfer als einen generell dynamischen evolutionären Prozess.

Eine Methode unabhängig von Annotationen in Datenbanken wurde für die Identifizierung der Intronpositionen entwickelt. Die analysierten charakteristischen Eigenschaften der spleißosomalen Introns in den hier untersuchten Genen zeigte eine sehr dynamische, Spezies spezifische Evolution von Introns. Die Introndichte und die Verteilung der Intronphasen, sowie eine überwiegende Anzahl von gemeinsamen Intronpositionen in Tieren und Pflanzen sind in Einklang mit Ergebnissen die aus Untersuchungen eukaryotischer Gene und Genome stammen. Es wurde keine Korrelation zwischen dem Zeitpunkt des Gentransfers und der Introndichte festgestellt, was auf eine hohe Rate neu eingefügter Introns nach der Integration eines Gens in das Wirtsgenom schließen lässt.

Ein eindeutiger Fall eines parallelen Introngewinns in Tieren und einer Grünalge wurde in dem Gen *nad7* identifiziert, welches unabhängig in diese Organismen transferiert wurde. Dieses Beispiel unterstützt die Meinung, daß gemeinsame Intronpositionen nicht unbedingt konserviert sind.

3 Introduction

3.1 Introns

DNA builds the genetic information of all living organisms except for some RNA viruses. Eukaryotic DNA contains a high fraction of regions which are not translated into protein sequences. Introns account for a large fraction of these non-coding regions and are usually composed of random sequences. If introns interrupt protein-coding genes in eukaryotes, the coding regions are called exons (Gilbert, 1978). The pre-mRNA (precurser messenger RNA) as the first product of transcription contains both, exons and introns. Before translation into a functional protein takes place, introns are excised from the transcript and the exonic regions are ligated. This process is called splicing and is one of the pre-mRNA processing mechanisms in eukaryotic cells.

Since their discovery in 1977 (Berget et al., 1977; Klessig, 1977; Chow et al., 1977) (Section 3.1.5.1), introns were the subject of many studies in which many of their characteristics were described (Section 3.1.1). Mechanistically different splicing processes define the two major groups of self-splicing introns and spliceosomal introns (Figure 3.1). The latter exist only in eukaryotic genomes and need a RNA-protein complex, the spliceosome (Grabowski et al., 1985; Brody and Abelson, 1985; Frendewey and Keller, 1985), to catalyze the splicing reaction, in contrast to the autocatalytic capabilities of the self-splicing introns. Questions about the possible function of introns in the genome or about the origin and evolution of spliceosomal introns are still under discussion.

3.1.1 Types of Introns

The different types of introns shown in Figure 3.1 grouped by their splicing mechanism into self-splicing and spliceosomal introns (Roy and Gilbert, 2006; Rodríguez-Trelles et al., 2006). Self-splicing introns contain group I and group II introns which both have ribozymic activity to catalyze their own excision (Pyle

and Lambowitz, 2006) but differ in the mechansim of the splicing reaction (Cech, 1990; Piccirilli, 2008; Tourasse and Kolstø, 2008). Both, group I and group II introns are found in bacterial and organellar genomes (Ferat and Michel, 1993). Group I introns which form the majority of self-splicing introns, are also found in the precursor ribosomal-RNA (pre-rRNA) in the nuclear genomes of protists and fungi (Harris and Rogers, 2008).

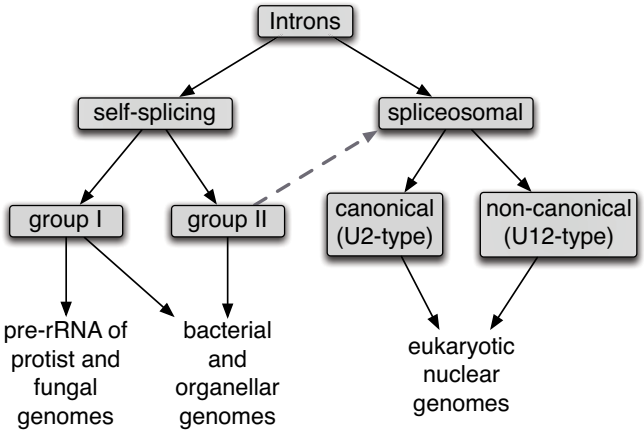


Figure 3.1: Types of introns are grouped according to their splicing mechanisms. Canonical and non-canonical introns are also referred to as U2 and U12 introns, respectively, named after the different components in the spliceosome. The dashed arrow indicates the possible evolutionary relationship between group II and spliceosomal introns, based on the mechanistical similarities between their splicing processes.

The occurrence of spliceosomal introns is restricted to eukaryotic nuclear genomes. They need the spliceosome to catalyze their excision from the pre-mRNA. The spliceosome is a RNA-protein complex which catalyzes the removal of introns in eukaryotes and comprises more than 200 proteins and five small nuclear RNAs (snRNAs) (Lamond, 1993; Valadkhan, 2007). More than 95% of the spliceosomal introns known so far, are canonical (U2-type) introns characterized by their highly conserved splicing sites GT-AG, which are recognized by the spliceosome. These canonical introns are excised from the pre-mRNA by the U2-dependent (major) spliceosome which is present in all eukaryotes. In contrast, the U12-dependent (minor) spliceosome (Jackson, 1991; Hall and Padgett, 1994) and the U12-type introns are not present in some organisms like yeast and nematodes (Burge et al., 1998). The snRNA composition of the major spliceosome comprises U1, U2, U4, U5 and U6 snRNAs (Burge et al., 1999; Jurica and Moore, 2003) whereas the minor spliceosome is composed of U11, U12, U4atac, U5 and

U6atac snRNAs (Tarn and Steitz, 1996; Will and Luhrmann, 2005). The splice sites of the U12-type introns have the consensus nucloetide pattern [A/G]T-A[C/G] and are not as strictly conserved as the GT-AG splice sites of the U2 introns. Because of similarities between the splicing procedures of self-splicing group II introns and spliceosomal introns, it is suggested that spliceosomal introns or components of the spliceosome, the snRNAs, might have originated from group II introns (Valadkhan, 2007; Toro et al., 2007; Toor et al., 2008). This probable evolutionary relationship between the different intron types is indicated in Figure 3.1.

3.1.2 Introns in protein coding genes

When a gene is transcribed, the resulting pre-mRNA usually undergoes several RNA-processing steps before the translation. These RNA-processing steps also include splicing, which catalyzes the removal of introns and the ligation of the two adjacent exons. Figure 3.2 illustrates a typical eukaryotic gene that contains an intron.

Variations in the splicing mechanism become manifested in the disruption of the common frameshift of a gene or in a different composition of exons. This process is called alternative splicing in which different mRNA transcripts arise from the same gene. A study within the ENCODE project, Encyclopedia of DNA elements (ENCODE, 2004), reports a high amount of alternative splice variants in the cell but it is not proven yet, if all of these proteins also have a function (Tress et al., 2007). A recent study confirms the high amount of splice variants in human, in which a tissue specific regulation of alternative splicing is reported (Wang et al., 2008).

As indicated in Figure 3.2, transcription and splicing are spatially divided from translation. Transcription and splicing are performed inside of the nucleus, the translation is accomplished outside of the nucleus. As a consequence, in eukaryotes there is no co-translation possible as in prokaryotes, in which the ribosomes start to translate the mRNA into amino acids while the transcription still continues. Co-translation would not be efficient in eukaryotes because the splicing process is slower than the translation. Here the nucleus prevents co-translation, because it separates transcription and splicing from translation (Martin and Koonin, 2006).

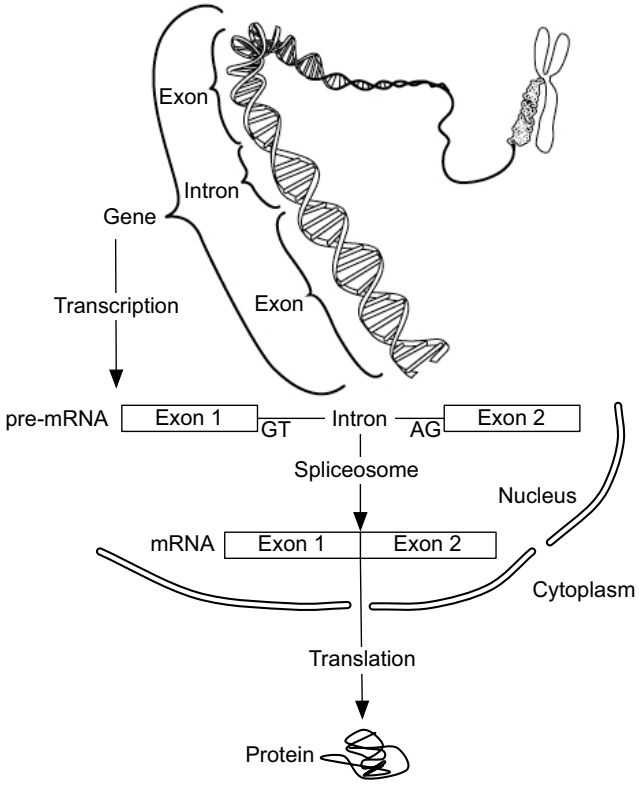


Figure 3.2: Illustration of a protein coding gene in eukaryotes. After transcription inside the nucleus, the pre-mRNA contains the two exons and the intron. The intron is spliced out of the pre-mRNA at the canonical splicing sites GT-AG and the two exons are rejoined, catalyzed by the spliceosome. The mRNA transcript is transported from the nucleus into the cytosol, where it is translated into a protein sequence.

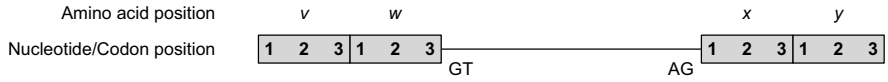
In eukaryotes, introns can also be found in non-coding regions of a gene, between the transcription initiation site and the translation initiation codon (Graur and Li, 2000), but in this work the focus is on spliceosomal introns in protein coding regions.

3.1.3 Intron characteristics

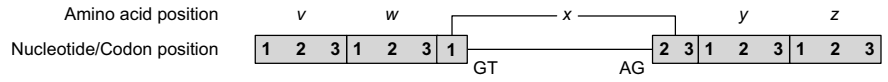
Spliceosomal introns within genes can be characterized and described by different features:

- number of introns per gene (intron density)
- intron position in the protein or nucleotide sequence (intron position)
- position of the intron in the codon (intron phase)
- length of the intron (intron length)

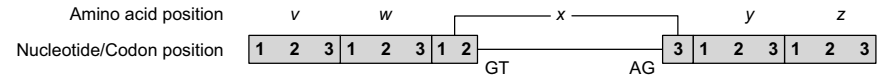
The length of introns varies between tens of bases up to hundreds of kilobases (Roy and Gilbert, 2006). Intron density can either be given as the number of introns in a gene or as the number of introns per basepairs of coding DNA. With the latter definition, the intron density can be compared among species. The position of an intron in a protein or a nucleotide sequence is given as the number of the amino acid and the nucleotide, respectively. The characteristic feature of the genetic code is that one amino acid is encoded by a triplet of nucleotides (codon). The position of an intron in the codon is described by the phase 0,1 or 2(Figure 3.3).



(a) Phase 0 intron



(b) Phase 1 intron

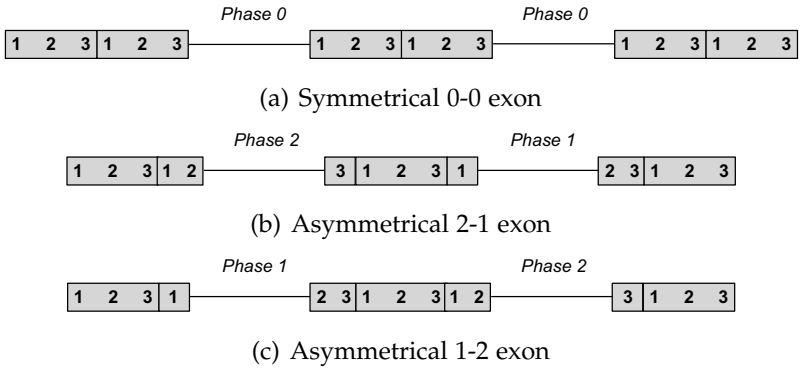


(c) Phase 2 intron

Figure 3.3: The intron phases. The intron is represented as a solid line and the canonical splicing sites GT-AG. The protein-coding regions are represented as gray boxes, separated into codons with the nucleotides 1, 2 and 3, which are later translated into the amino acids *v, w, x, y* and *z*. In all three cases, the intron position is at site *x* in the protein sequence.

If the intron is located between two codons it is of phase zero (Figure 3.3(a)). If the intron is located between the first and the second or the second and the third nucleotide of a codon, the intron is of phase one or two, respectively (Figure 3.3(b), 3.3(c)). The phases of the introns lead to different classes of exons. Exons that are flanked by two introns of the same phase are symmetrical exons, otherwise they are asymmetrical. Limited by three possible intron phases, there are nine different patterns of exons. Three possible symmetrical exons (0-0, 1-1 and 2-2 exons) and six possible asymmetrical exons (0-1, 0-2, 1-0, 1-2, 2-0 and 2-1 exons). Figure 3.4(a) represents a 0-0 exon which is flanked by two introns of phase 0. Figure 3.4(b) shows a 2-1 exon as an example for an asymmetrical exon. To emphasize that there is a difference between the order of intron phases

which surround an exon, a 1-2 exon is shown in Figure 3.4(c).



(a) Symmetrical 0-0 exon

(b) Asymmetrical 2-1 exon

(c) Asymmetrical 1-2 exon

Figure 3.4: The symmetry of exons is illustrated by three examples. One symmetrical exon (a) out of three possible symmetrical exons and two asymmetrical exons (b, c) out of six possible asymmetrical exons are shown to emphasize that there is a difference between a 2-1 and a 1-2 asymmetrical exons (as well as between 0-1, 1-0 and 0-2, 2-0 exons).

The exon symmetry point out to restrictions regarding to a process called exon shuffling (Gilbert, 1978; Williamson, 1977). Exon shuffling produces new combinations of exons through intergenic recombination of introns, leading to the evolution of new functions (Patthy, 1987, 1999; Roy, 2003). The insertion of an exon into an intron region as an important mechanism of exon shuffling for example, will only be successful if the reading frame of the transcript is still retained.

3.1.4 Distribution of introns among eukaryotes

The overall intron density in eukaryotic genomes varies considerably. Figure 3.5 shows the average number of introns per gene for various eukaryotes, sorted by ascending intron density. The human and mouse genomes have the highest amount of introns, followed by two higher plants *Arabidopsis thaliana* and *Oryza sativa*. The species *Candida albicans* and *Saccharomyces cerevisiae* are known to have low intron densities, whereas another yeast species *Schizosaccharomyces pombe* contains a much higher amount of introns in the nuclear genome. The first six species in the diagram of Figure 3.5 seem to lack introns in their genomes completely (*Leishmania major, Giardia lamblia, Trichomonas vaginalis, Cyanidioschyzon merolae, Encephalitozoon cuniculi, Cryptosporidium parvum*). But at least few

introns were found in *Giardia lamblia* (Nixon et al., 2002; Russell et al., 2005) and *Trichomonas vaginalis* (Vanácová et al., 2005; Carlton et al., 2007) for example. Especially the finding of spliceosomal introns in the deep-branching parabasalid *Trichomonas vaginalis* leads to the assumption that most probably all eukaryotic genomes accomodate spliceosomal introns because they were already present in early eukaryotic evolution (Vanácová et al., 2005). These differences in the number of introns among eukaryotes, cannot be explained by either the phylogenetic relationships, the genome dimension or the complexity of the organisms (Roy and Gilbert, 2006; Rodríguez-Trelles et al., 2006).

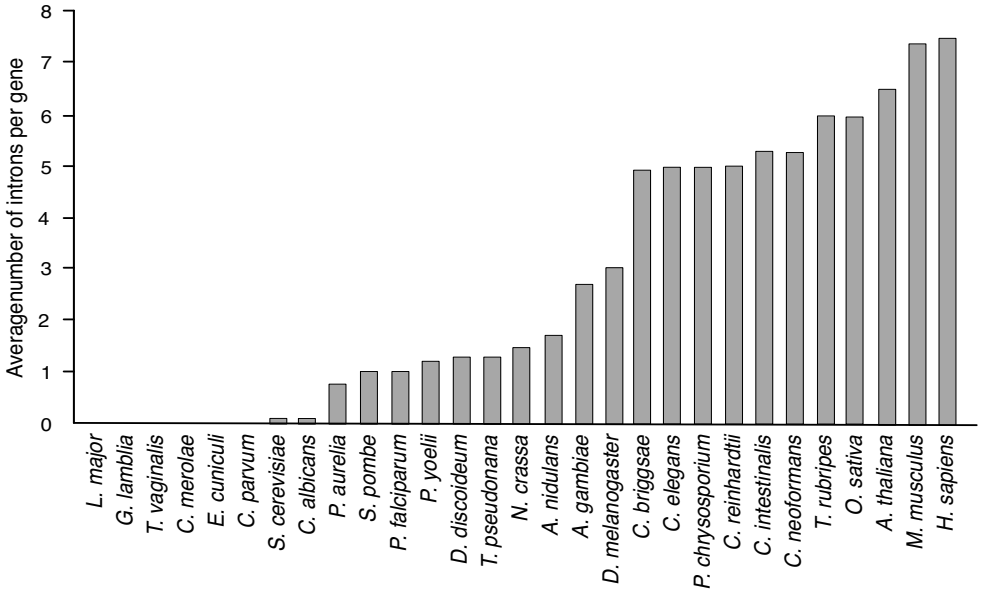


Figure 3.5: Intron density among eukaryotes, based on Roy and Gilbert (2006). The intron density (here: average number of introns per gene) is plotted for different eukaryotes. From left to right: *Leishmania major, Giardia lamblia, Trichomonas vaginalis, Cyanidioschyzon merolae, Encephalitozoon cuniculi, Cryptosporidium parvum, Saccharomyces cerevisiae, Candida albicans, Paramecium aurelia, Schizosaccharomyces pombe, Plasmodium falciparum, Plasmodium yoelii, Dictyostelium discoideum, Thalassiosira pseudonana, Neurospora crassa, Aspergillus nidulans, Anopheles gambiae, Drosophila melanogaster, Caenorhabditis briggsae, Caenorhabditis elegans, Phanerochaete chrysosporium, Chlamydomonas reinhardtii, Ciona intestinalis, Cryptococcus neoformans, Takifugu rubripes, Oryza sativa, Arabidopsis thaliana, Mus musculus, Homo sapiens*.

3.1.5 Evolutionary origin of introns

3.1.5.1 Early views on intron evolution

Introns were first observed and described in the literature in several different studies in 1977 where they were discovered in the mRNA of the human adenovirus 2 (Berget et al., 1977; Klessig, 1977; Chow et al., 1977; Sambrook, 1977; Aloni et al., 1977) and in ovalbumin, β-globin and immunoglobulin genes of animals (Breathnach et al., 1977; Doel et al., 1977; Jeffreys and Flavell, 1977; Brack and Tonegawa, 1977; Williamson, 1977). The conception of a monocistronic gene structure was disproved with these findings. Gilbert proposed the name introns (= intragenic regions) for the sequences which split the genes, in contrast to exons (= expressed regions) (Gilbert, 1978). He also predicted introns as a large source of new genetic functions through exon shuffling (Section 3.1.1). Because of the non-coding regions between exons, the duplication, insertion or deletion of exons could result in new proteins.

Spliceosomal introns and the spliceosomal machinery have been identified in all eukaryotic genomes but they are absent from all prokaryotic genomes sequenced to date (Collins and Penny, 2005). Aside from similarities between the group II self-splicing introns and the spliceosome (Valadkhan, 2007; Toro et al., 2007; Toor et al., 2008) (Section 3.1.1), no transitional form between self-splicing introns and spliceosomal introns could be identified. The influences of introns on the evolution of eukaryotic genes, selective forces and new evolving protein functions by alternative splicing still take influence on our understanding and the definition of what exactly a gene is (Pesole, 2008).

3.1.5.2 Origin of spliceosomal introns

There are many open questions about the origin of spliceosomal introns. Especially the questions "when" and "where" they originated lead to different hypotheses and scenarios about their evolution and their contribution to the genome structure we see today. The origin of spliceosomal introns is mainly discussed in the context of two different hypotheses.

The introns-early hypothesis states that spliceosomal introns arose early in evolution of the genome, so introns were present in the last common ancestor of prokaryotes and eukaryotes (Darnell, 1978; Doolittle, 1978; Gilbert, 1978). In this hypothesis, spliceosomal introns were subsequently lost in all prokaryotes to

explain their absence in these genomes. In contrast, the introns-late hypothesis states that spliceosomal introns originated after the divergence of prokaryotes and eukaryotes (Cavalier-Smith, 1985; Palmer and Logsdon, 1991). Furthermore the introns-late hypothesis links the origin of spliceosomal introns to the origin of mitochondria in eukaryotes (Cavalier-Smith, 1991; Martin and Koonin, 2006). The introns-early and introns-late hypotheses are still debated in the literature (Rogozin et al., 2005; Belshaw and Bensasson, 2006; Roy and Gilbert, 2006). With every newly sequenced genome more information is added to our understanding of intron evolution throughout eukaryotic lineages. The different hypotheses are both supported until now, because the observations and results of exon/intron structure in the light of the general knowledge of genomic evolution do not unambigously reject one of them. Combining different theoretical scenarios based on logical assumptions with results of comparative genomic or experimental analyses, the different hypotheses try to explain the origin of spliceosomal introns, while each hypothesis builds a framework which includes the observations and makes predictions which can be tested. Differences in intron density among different species for example, are mostly explained as a loss of introns rather than a species specific development. The ancient origin of introns is supported by the observation of shared intron positions among eukaryotes, especially if they are found within divergent lineages. These introns should reflect a conservation of the position at which the ancient gain of the intron happend before speciation events (Gilbert and Glynias, 1993).

Dynamics of intron gain and loss are used as a resource to deduce information about the evolution of introns (Roy and Gilbert, 2005; Irimia and Roy, 2008). Intron gain and loss is a very controversial subject in which no clear trend or general evolutionary mechanism can be defined for all eukaryotes yet (Sharpton et al., 2008). For example, there is a general low intron gain and loss rate observed in *Plasmodium falciparum* (Roy and Hartl, 2006) but a high rate of intron gain and loss events identified in *Arabidopsis thaliana* (Knowles and McLysaght, 2006). There exist five different models of how an intron can be gained (Roy and Gilbert, 2006; Rogers, 1989):

- Intron transposition
- Transposon insertion
- Tandem genomic duplication
- Intron transfer

- Self-splicing group II introns

Intron loss can be explained with two possible mechanisms (Roy and Gilbert, 2006; Mourier, 2005; Lewin, 1983):

- reverse transcribed copy of a spliced mRNA transcript recombines with the genomic copy
- genomic deletion

According to the introns-late theory, there is evidence to suggest a connection between the evolution of spliceosomal introns and self-splicing group II introns as indicated in Figure 3.1. Because of the ability of group II introns to function as mobile retroelements they were suggested to invade DNA through reverse splicing reactions, but they themselves bear an indistinct evolutionary history as well (Zimmerly et al., 1995; Zimmerly and Hausner, 2001). The possibility of a common origin of spliceosomal introns and group II introns is becoming more significant with new observations and insights into the complex mechanisms and molecular structures of self-splicing and the spliceosome (Lambowitz and Zimmerly, 2004; Robart et al., 2007; Valadkhan, 2007; Toro et al., 2007; Toor et al., 2008).

3.2 Evolutionary origin of mitochondria

Mitochondria are the main energy suppplier of eukaryotic cells. The cell organelles were once free living prokaryotes and originated by endosymbiosis (Gray et al., 1999). This endosymbiosis started with the engulfment of a facultative anaerobic α-proteobacterium by a host cell. Through this endosymbiotic event, the prokaryotic endosymbionts developed into cell organelles in their host cells (Martin et al., 2001).

The hypothesis of endosymbiosis for the origin of plastids was first developed in the literature in 1905 by Mereschkowsky (Mereschkowsky, 1905). The first time that mitochondria were described to have a bacterial origin was in 1927 (Wallin, 1927) but the hypothesis was not accepted as an explanation for the origin of these organelles at that time. Later, the endosymbiotic theory and the bacterial origin of mitochondria appeared in the literature again (Sagan, 1967; Goksøyr, 1967) and finally got serious acceptation in the scientific community.

α-proteobacteria were revealed as the prokaryotic group most closely re-

lated to mitochondria and the ancestors of these organelles (Gray et al., 2001; Wu et al., 2004). The α-proteobacteria *Rickettsia prowazekii* was suggested to represent the mitochondrial ancestor (Andersson et al., 1998; Emelyanov, 2003), but problems in this result are reported in other studies, regarding to insufficiency of phylogenetic methods and the constitution of recent α-proteobacterial genomes, which do not exactly represent the ancestral mitochondrion (Esser et al., 2004, 2007). Another way to approach the state of the ancestral mitochondrion without defining the most closely related species of α-proteobacteria was for example, to reconstruct the proto-mitochondrial metabolism (Gabaldón and Huynen, 2003).

Comparably to the question about the ancestor of mitochondria, the endosymbiotic theory opened the question about the nature of the host cell. Various hypotheses exist with the main difference that either the host cell already was a primitive eukaryote (Moreira and Lopez-Garcia, 1998; Cavalier-Smith, 2002, 2004; Margulis et al., 2005), or it was an archaebacterial-like prokaryote, in which the endosymbiotic event initiated the evolution of eukaryotes (Martin and Müller, 1998; Vellai et al., 1998; Martin et al., 2003; Embley and Martin, 2006). All present known eukaryotes possess mitochondria or variations of them, the mitosomes and hydrogenosomes, which have the same origin as mitochondria. The organisms *Giardia* and *Trichomonas* for example, were thought to be amitochondrial eukaryotes, before it became clear that *Giardia* possesses mitosomes (Tovar et al., 1999, 2003) and *Trichomonas* hydrogenosomes (Lindmark and Müller, 1973; Martin and Müller, 1998).

3.2.1 Endosymbiotic gene transfer

Some of the influences of the mitochondrion on the eukaryotic evolution may be evident, as the production of energy for the cell for example (Section 3.2.1.1). Comparative sequence analyses allowed to trace the origin of mitochondria to α-proteobacteria (Section 3.2), and also led to the discovery of endosymbiotic gene transfer (Martin et al., 1998; Martin and Herrmann, 1998; Timmis et al., 2004). This process defines the transfer of genes from the endosymbiont to the host genome. Integration of genes into the host nucleus and loss of those genes in the organellar genomes are the main reason for the reduced organellar genome. Some genes were also lost completely during the evolution of eukaryotes (Fig-

ure 3.6).

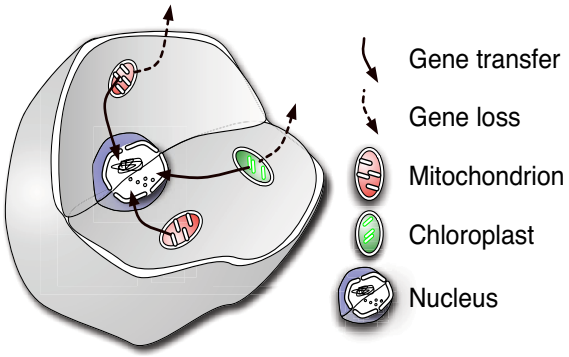


Figure 3.6: Endosymbiotic gene transfer of organellar genes to the nuclear genome. Genes of mitochondria (red) and chloroplasts (green) can be transferred into the nucleus of the host (solid arrows), but genes can also be lost from the organellar genome (dashed arrow) during the evolution of eukaryotic cells.

Figure 3.6 shows the endosymbiotic gene transfer of mitochondria and chloroplasts and indicates gene loss. The photosynthetic chloroplasts, found in plants and algae, originated by endosymbiosis, like mitochondria. The ancestry of chloroplast lies within the cyanobacteria (Goksøyr, 1967), but a specific lineage of cyanobacteria that gave rise to chloroplasts could first not be identified (Douglas, 1998; Delwiche, 1999). With growing number of available plant and cyanobacterial genomes the amount of genes that were transferred from the chloroplast to the nuclear genome of *Arabidopsis thaliana* was inferred to be between 2-9% with *Synechocystis* as the most representative ancestor of chloroplasts (Rujan and Martin, 2001). Including more genome sequences to the analyses gave a rise up to 18% of chloroplast genes in *Arabidopsis* (Martin et al., 2002) and 14% among four different plant genomes, indicating heterocyst-forming species as the cyanobacteria which seem to be most similar to the ancestor of chloroplasts (Deusch et al., 2008).

Different evolutionary scenarios have to be considered for the identification of genes originated by endosymbiotic gene transfer. Genes that were transferred to the host genome and evolved to have a new function might only expose their origin in phylogenetic analyses (Gabaldón and Huynen, 2003), while genes that have a function in the organelle did not necessarily originate from the bacterial ancestor of the organelle (Section 3.2.1.1).

3.2.1.1 Oxidative phosphorylation pathway

The oxidative phosphorylation pathway is a key process of mitochondria in which ATP is produced, the major energy source in the cell (Figure 3.7).

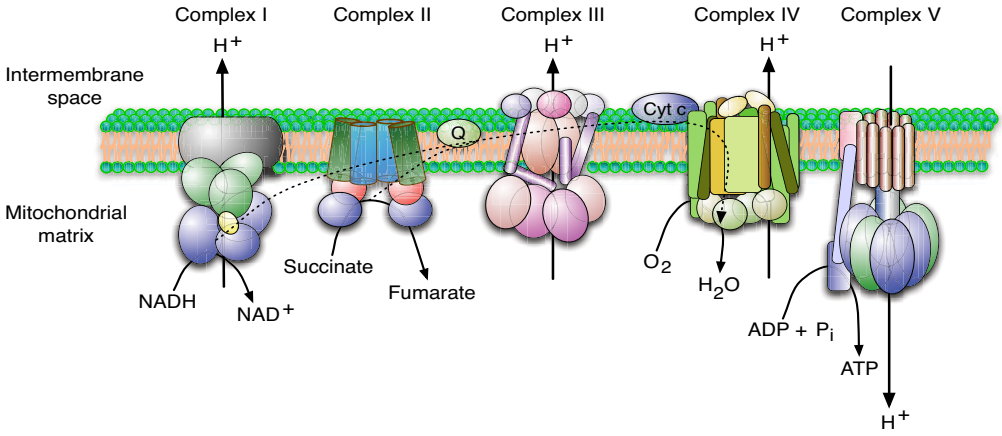


Figure 3.7: Oxidative phosphorylation pathway. The five protein complexes are embedded into the mitochondrial inner membrane. The pathway consists of the electron transport chain which includes four protein complexes (Complex I – NADH-ubiquinone oxidoreductase; Complex II – Succinate-ubiquinone oxidoreductase; Complex III – Ubiquinol-cytochrome c oxidoreductase; Complex IV – Cytochrome c oxidase) and the ATP Synthase (Complex V). The flow of electrons is shown by the dotted arrows, with two possible electron sources NADH or succinate and is supported by the electron shuttle molecules Coenzyme Q (Q) and cytochrome c (Cyt c). Oxygen serves as the final electron acceptor. Protons (H^+) are released into the intermembrane space. The resulting proton gradient is used by the Complex V to produce ATP. (based on http://www.genome.ad.jp/kegg/pathway/map/map00190.html)

Oxidative phosphorylation consists of the electron transport chain (Complex I-IV) including two electron shuttle molecules (Coenzyme Q and Cytochrome c) and the ATP-synthase (Complex V) which are all located in the mitochondrial inner membrane. The genes for these proteins are encoded either in the mitochondrial or in the nuclear genome. The proteins are organized within five multiprotein complexes (Saraste, 1999), up to approximately 113 proteins in human mitochondria (van Waveren and Moraes, 2008). Electrons are transferred via the redox groups of the complexes to the final acceptor oxygen (Complex IV). The resulting free energy is used to pump protons out of the mitochondrial matrix into the intermembrane space, which generates an electrochemical gradient across the mitochondrial inner membrane. Proton flux back into the mitochondrial matrix is going through Complex V and is coupled with ATP synthesis.

The first proposed principle of this mechanism was first described by Mitchell (1961, 1979) who explained with the chemiosmotic theory the linkage between respiration and ATP synthesis in the mitochondrion.

A high similarity is found between mitochondrial proteins of the electron transport chain and those of α-proteobacteria, specifically *Rickettsia prowazekii*. The main difference of the bacterial electron transport chain is that bacteria can use many different electron donors and electron acceptors, allowing prokaryotes to grow under various environmental conditions. The most studied prokaryotic oxidative phosphorylation pathway is that of the bacterium *Escherichia coli* (Ingledew and Poole, 1984).

3.2.1.2 Mitochondrial ribosomal proteins

Mitochondria posess their own circular DNA and their own mitochondrial ribosomes. These 55S mitoribosomes are differ in protein and rRNA composition from both, prokaryotic and eukaryotic ribosomes (Table 3.1). Despite of the varying composition, all three types of ribosomes perform the same task, namely the translation of a gene sequence into a polypeptide chain as the second step in protein biosynthesis. In all ribosomes, the small subunit binds the mRNA and the large subunit catalyzes the polypeptide elongation.

Table 3.1: Different composition of prokaryotic, eukaryotic and mitochondrial ribosomes. The rRNA composition and the number of proteins in each subunit are listed.

	Mitochondria	Prokaryotes	Eukaryotes
Functional ribosome	**55S**	**70S**	**80S**
Large subunit	39S	50S	60S
rRNA	16S	23S 5S	28S 5.8S 5S
proteins	48	34	49
Small subunit	28S	30S	40S
rRNA	12S	16S	18S
proteins	29	31	33

The mitochondrial rRNAs are encoded in the mitochondrial genome (Anderson et al., 1981). In contrast, most of the mitochondrial ribosomal proteins are encoded in the nucleus and synthesized in the cytosol of the cell (Schieber and

O Brien, 1985). Assembly of the mitochondrial ribosomes take place after the transfer of the proteins into the mitchondrion.

3.3 Goals of this study

The goal of this study was to gain insight into the evolution of spliceosomal introns within nuclear genes that originated by endosymbiotic gene transfer from the mitochondrion. In accordance with the introns-late hypothesis, spliceosomal introns were supposed to originate during the evolution of eukaryotes, with a common ancestry of self-splicing group II introns (Cavalier-Smith, 1985; Cech, 1986), which is supported by similarities between splicing mechanisms of spliceosomal introns and those of group II introns (Valadkhan, 2007; Toor et al., 2008). The fact that group II introns are found in bacterial and mitochondrial genomes, suggests an evolutionary connection between spliceosomal introns and the origin of mitochondria (Cavalier-Smith, 1991; Martin and Koonin, 2006), and leads to study the dynamics of intron evolution in eukaryotic genes that originated from endosymbiotic gene transfer from mitochondria.

Genes that originated by endosymbiotic gene transfer did not contain spliceosomal introns when they were transferred to the host genome. Nuclear encoded genes with mitochondrial origin can be identified with sequence similarity to their α-proteobacterial homologs (Martin et al., 2001; Esser et al., 2004). Intron characteristics, like intron density, phase distribution and shared intron positions in these genes should provide insights into the dynamics of intron evolution in eukaryotes.

4 Material and Methods

4.1 Sequence data

Completely sequenced genomes of various eukaryotes were downloaded from public databases, including genomes of plants and green algae (*Arabidopsis thaliana*, *Oryza sativa*, *Chlamydomonas reinhardtii*) a diatom (*Thalassiosira pseudonana*), two parasitic protists (*Plasmodium falciparum*, *Leishmania major*), a mycetozoa (*Dictyostelium discoideum*), five fungi (*Aspergillus fumigatus*, *Candida glabrata*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*) and six animals (*Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*). These 18 organisms were chosen to provide a representative set of genomes across eukaryotes, including the most diverse multicellular eukaryotes plants, fungi and animals. For each genome, the corresponding set of proteins was downloaded, too. The database sources of the sequences are listed in Table 4.1. Either the sequences were received in a single file, if the whole chromosome or protein set could be downloaded all at once, or the sequences of the single chromosomes were downloaded via ftp using a shell-script and later assembled into one dataset for the corresponding species. The sequences were stored as local nucleotide and protein databases.

Table 4.1: Sources of genome and protein sequences. Most of the sequences could be downloaded from NCBI as a full set of sequences. The URLs are listed beneath the table.

Organism	Genome sequences	Protein sequences
Arabidopsis thaliana	NCBI	NCBI
Chlamydomonas reinhardtii	JGI	JGI
Oryza sativa	EBI (Integr8)	NCBI (RefSeq)
Dictyostelium discoideum	EBI (Integr8)	NCBI (RefSeq)
Leishmania major	Sanger Institute	Sanger Institute
Plasmodium falciparum	NCBI	NCBI
Thalassiosira pseudonana	JGI	JGI
Aspergillus fumigatus	NCBI	NCBI
Candida glabrata	NCBI	NCBI
Saccharomyces cerevisiae	NCBI	NCBI
Schizosaccharomyces pombe	NCBI	NCBI
Yarrowia lipolytica	NCBI	NCBI
Danio rerio	NCBI	NCBI
Drosophila melanogaster	NCBI	NCBI
Caenorhabditis elegans	NCBI	NCBI
Homo sapiens	NCBI	NCBI
Mus musculus	NCBI	NCBI
Rattus norvegicus	NCBI	NCBI

NCBI – http://www.ncbi.nlm.nih.gov/(03/2007)
JGI – http://www.jgi.doe.gov/(03/2007)
EBI – http://www.ebi.ac.uk/(03/2007)
Sanger Institute – http://www.sanger.ac.uk/(03/2007)

4.1.1 Identifying the α-proteobacterial origin of genes

Nuclear encoded genes of *Homo sapiens* of the oxidative phosphorylation pathway as well as the mitochondrial ribosomal proteins were received from the SWISS-PROT-database[1] (Boeckmann et al., 2003). The proto-mitochondrial origin of the genes of the oxidative phosphorylation pathway was tested as shown in Figure 4.1.

[1] http://www.ebi.ac.uk/swissprot/

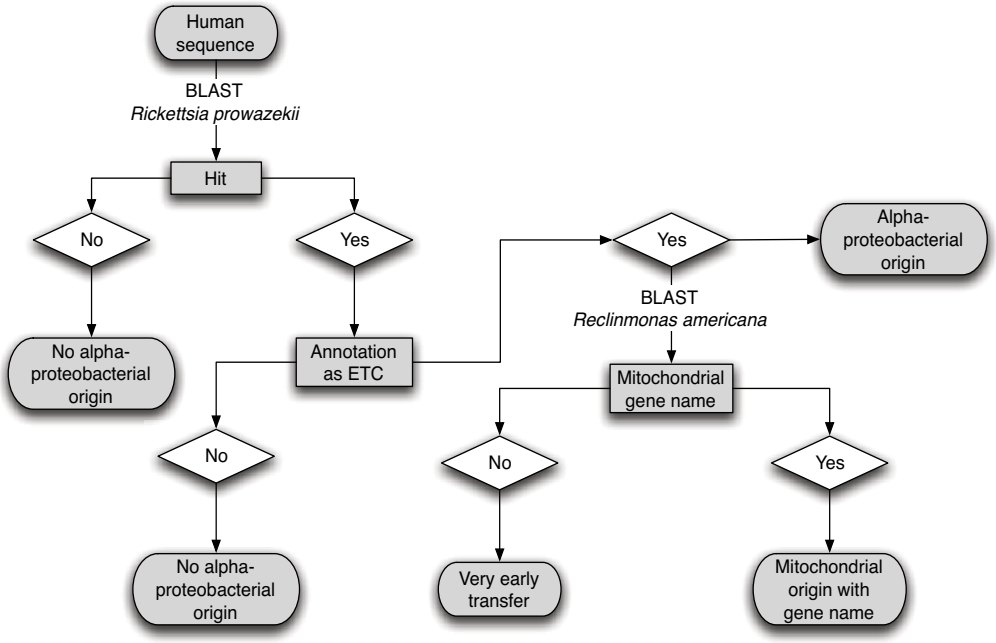


Figure 4.1: Identification of the proteins of α-proteobacterial origin and specification of their mitochondrial gene names, if possible.

The human genes listed in Table 4.2 - Table 4.8 were used as query sequences for a homology search in the protein database of the 18 eukaryotes (Section 4.1.2). With each protein sequence a BLAST search (Altschul et al., 1997) was performed against the genome of the α-proteobacterium *Rickettsia prowazekii*. The oxidative phosphorylation pathway in *Rickettsia prowazekii* is highly similar to the pathway in aerobic mitochondria (Müller and Martin, 1999). If the search resulted in a hit, annotated with a function in the electron transport chain, a second BLAST search was performed against the genome of the protozoon *Reclinomonas americana*. The mitochondria of *Reclinomonas americana* contain the biggest mitochondrial genome, representing a miniature eubacterial genome (Lang et al., 1997) which can be used to infer the gene names of the human proto-mitochondrial genes. The swissprot identifier of all human query proteins are listed in Table 4.2 - Table 4.8. For the mitochondrial ribosomal proteins this information was taken from Smits et al. (2007) where all mitochondrial ribosomal proteins were chosen which have a homologous sequence in *Rickettsia prowazekii*. With information about an existing homolog in *Reclinomonas americana* the mitochondrial gene name could be assigned to the nuclear encoded proteins as described for the genes of the oxidative phosphorylation pathway.

Table 4.2: Proteins of the oxidative phosphorylation pathway, in *Homo sapiens*, Complex I (NADH-ubiquinone oxidoreductase) are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **NADH dehydrogenase [ubiquinone]...**
O00217	iron-sulfur protein 8, mitochondrial [Precursor]
O00483	1 alpha subcomplex subunit 4
O15239	1 alpha subcomplex subunit 1
O43181	iron-sulfur protein 4, mitochondrial [Precursor]
O43674	1 beta subcomplex subunit 5, mitochondrial [Precursor]
O43676	1 beta subcomplex subunit 3
O43677	subunit C1, mitochondrial [Precursor]
O43678	1 alpha subcomplex subunit 2
O43920	iron-sulfur protein 5
O75251	iron-sulfur protein 7, mitochondrial [Precursor]
O75306	iron-sulfur protein 2, mitochondrial [Precursor]
O75380	iron-sulfur protein 6, mitochondrial [Precursor]
O75438	1 beta subcomplex subunit 1
O75489	iron-sulfur protein 3, mitochondrial [Precursor]
O95139	1 beta subcomplex subunit 6
O95167	1 alpha subcomplex subunit 3
O95168	1 beta subcomplex subunit 4
O95169	1 beta subcomplex subunit 8, mitochondrial [Precursor]
O95178	1 beta subcomplex subunit 2, mitochondrial [Precursor]
O95182	1 alpha subcomplex subunit 7
O95298	1 subunit C2
O95299	1 alpha subcomplex subunit 10, mitochondrial [Precursor]
O96000	1 beta subcomplex subunit 10
P17568	1 beta subcomplex subunit 7
P19404	flavoprotein 2, mitochondrial [Precursor]
P28331	75 kDa subunit, mitochondrial [Precursor]
P49821	flavoprotein 1, mitochondrial [Precursor]
P51970	1 alpha subcomplex subunit 8
P56181	flavoprotein 3, mitochondrial [Precursor]
P56556	1 alpha subcomplex subunit 6
Q16718	1 alpha subcomplex subunit 5
Q16795	1 alpha subcomplex subunit 9, mitochondrial [Precursor]
Q86Y39	1 alpha subcomplex subunit 11
Q9NX14	1 beta subcomplex subunit 11, mitochondrial [Precursor]
Q9P0J0	1 alpha subcomplex subunit 13
Q9UI09	1 alpha subcomplex subunit 12
Q9Y6M9	1 beta subcomplex subunit 9

Table 4.3: Proteins of the oxidative phosphorylation pathway in *Homo sapiens*, Complex II (succinate dehydrogenase-CoQ oxidoreductase) are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **Succinate dehydrogenase [ubiquinone]...**
O14521	cytochrome b small subunit, mitochondrial [Precursor]
P21912	iron-sulfur subunit, mitochondrial [Precursor]
P31040	flavoprotein subunit, mitochondrial [Precursor]
Q99643	cytochrome b560 subunit, mitochondrial [Precursor]

Table 4.4: Proteins of the oxidative phosphorylation pathway in *Homo sapiens*, Complex III (cytochrome reductase) are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **Cytochrome...**
O14949	b-c1 complex subunit 8
O14957	b-c1 complex subunit 10
P07919	b-c1 complex subunit 6, mitochondrial [Precursor]
P08574	c1, heme protein, mitochondrial [Precursor]
P14927	b-c1 complex subunit 7
P22695	b-c1 complex subunit 2, mitochondrial [Precursor]
P31930	b-c1 complex subunit 1, mitochondrial [Precursor]
P47985	b-c1 complex subunit Rieske, mitochondrial [Precursor]
Q9UDW1	b-c1 complex subunit 9

Table 4.5: Proteins of the oxidative phosphorylation pathway in *Homo sapiens*, Complex VI (cytochrome oxidase) are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **Cytochrome c oxidase...**
O14548	subunit 7A-related protein, mitochondrial [Precursor]
O60397	subunit 7A3, mitochondrial [Precursor]
P09669	polypeptide VIc [Precursor]
P10176	polypeptide 8A, mitochondrial [Precursor]
P10606	subunit 5B, mitochondrial [Precursor]
P12074	polypeptide 6A1, mitochondrial [Precursor]
P13073	subunit 4 isoform 1, mitochondrial [Precursor]
P14406	polypeptide 7A2, mitochondrial [Precursor]
P14854	subunit VIb isoform 1
P15954	subunit 7C, mitochondrial [Precursor]
P20674	subunit 5A, mitochondrial [Precursor]
P24310	polypeptide 7A1, mitochondrial [Precursor]
P24311	polypeptide 7B, mitochondrial [Precursor]
P99999	Cytochrome c
Q02221	polypeptide 6A2, mitochondrial [Precursor]
Q6YFQ2	subunit VIb isoform 2
Q7Z4L0	polypeptide 8C, mitochondrial [Precursor]
Q8TF08	polypeptide 7B2, mitochondrial [Precursor]
Q96KJ9	subunit 4 isoform 2, mitochondrial [Precursor]

Table 4.6: Proteins of the oxidative phosphorylation pathway in *Homo sapiens*, Complex V (ATP synthase) are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein namem, **ATP synthase...**
O75947	subunit d, mitochondrial
O75964	subunit g, mitochondrial
P05496	lipid-binding protein, mitochondrial [Precursor]
P06576	subunit beta, mitochondrial [Precursor]
P18859	coupling factor 6, mitochondrial [Precursor]
P24539	subunit b, mitochondrial [Precursor]
P25705	subunit alpha, mitochondrial [Precursor]
P30049	subunit delta, mitochondrial [Precursor]
P36542	subunit gamma, mitochondrial [Precursor]
P48047	subunit O, mitochondrial [Precursor]
P48201	lipid-binding protein, mitochondrial [Precursor]
P56134	subunit f, mitochondrial
P56381	subunit epsilon, mitochondrial
P56385	subunit e, mitochondrial
Q06055	lipid-binding protein, mitochondrial [Precursor]
Q7Z4Y8	subunit g 2, mitochondrial

Table 4.7: Proteins of the mitochondrial ribosome in *Homo sapiens*, large subunit are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **39S ribosomal protein...**
O75394	L33, mitochondrial
P09001	L3, mitochondrial
P49406	L19, mitochondrial [Precursor]
Q13084	L28, mitochondrial [Precursor]
Q16540	L23, mitochondrial
P52815	L12, mitochondrial [Precursor]
Q7Z2W9	L21, mitochondrial [Precursor]
Q7Z7H8	L10, mitochondrial [Precursor]
Q6P1L8	L14, mitochondrial [Precursor]
Q8TCC3	L30, mitochondrial [Precursor]
Q96A35	L24, mitochondrial [Precursor]
Q5T653	L2, mitochondrial [Precursor]
Q9BQ48	L34, mitochondrial [Precursor]
Q9BRJ2	L45, mitochondrial [Precursor]
Q9BYC8	L32, mitochondrial [Precursor]
Q9BYC9	L20, mitochondrial [Precursor]
Q9BYD1	L13, mitochondrial
Q9BYD2	L9, mitochondrial [Precursor]
Q9BYD3	L4, mitochondrial
Q9BYD6	L1, mitochondrial [Precursor]
Q9HD33	L47, mitochondrial [Precursor]
Q9H0U6	L18, mitochondrial [Precursor]
Q9NRX2	L17, mitochondrial [Precursor]
Q9NWU5	L22, mitochondrial [Precursor]
Q9NX20	L16, mitochondrial [Precursor]
Q9NZE8	L35, mitochondrial [Precursor]
Q9P015	L15, mitochondrial [Precursor]
Q9P0J6	L36, mitochondrial [Precursor]
Q9P0M9	L27, mitochondrial
Q9Y3B7	L11, mitochondrial [Precursor]

Table 4.8: Proteins of the mitochondrial ribosome in *Homo sapiens*, small subunit are listed with their SWISS-PROT identifiers and the corresponding protein names.

SWISS-PROT ID	Protein name, **28S ribosomal protein...**
O15235	S12, mitochondrial [Precursor]
O60783	S14, mitochondrial
P82664	S10, mitochondrial
P82675	S5, mitochondrial
P82912	S11, mitochondrial [Precursor]
P82914	S15, mitochondrial [Precursor]
P82921	S21, mitochondrial
P82932	S6, mitochondrial
P82933	S9, mitochondrial [Precursor]
Q96EL2	S24, mitochondrial [Precursor]
Q9Y2R5	S17, mitochondrial [Precursor]
Q9Y2R9	S7, mitochondrial [Precursor]
Q9Y399	S2, mitochondrial
Q9Y3D3	S16, mitochondrial [Precursor]

4.1.2 Homology search and multiple alignments

To find homologs of the human proto-mitochondrial proteins in the set of 18 eukaryotic genomes, a protein BLAST (Altschul et al., 1997) (BLASTP) was performed with each sequence. All BLAST hits with an e-value threshold of 10^{-06} were taken as a set of homologous sequences. To each set the query sequence was added. If the sequence itself was found as the best hit in *Homo sapiens* it was excluded from further analyses but other human homologous proteins were accepted. Thus all species can be represented by more than one sequence in one dataset. For each set of homologous sequences multiple sequence alignments were computed with MUSCLE (Edgar, 2004b,a). Two different output file formats of the alignments were generated: the CLUSTAL W format was used to visualize the alignment in html and was reformatted into phylip format for reconstructing phylogenetic trees (Section 4.9).

4.2 Identifying intron positions

4.2.1 Exon-intron databases

In the first approach, information about intron positions was obtained from pre-existing exon-intron databases. The exon-intron databases described in this section are secondary databases, meaning that they based on the data of primary databases. The Xpro database (Gopalan et al., 2004) for example processes the data of one of the most comprehensive public databases, GenBank (Benson et al., 2008). It takes the information on annotated exon/intron gene structures in GenBank and stores additional data like the splicing sites and intron phases. In the Xpro database, intron positions are validated by aligning the genomic sequence with EST sequences found in EST-databases using the program BLAT (Kent, 2002). With this validation alternative splicing variants are identified.

The ExInt database (Sakharkar et al., 2000, 2002) stores the information in a relational database (MySQL) in addition to a file in text-format. It is also based on annotated sequences in GenBank. In consideration of redundancy found in GenBank, the authors created a non-redundant dataset avoiding a bias in their database statistics. The IDB (Intron DataBase) and IEDB (Intron Evolution DataBase) (Schisler and Palmer, 2000)) contain additional data from the SWISS-PROT database. Exon/intron structures are also stored in this database. Moreover the IEDB provides statistical information about the sequences included in IDB, for example intron density and distribution of introns in the species.

In the first approach, the EID (Exon-Intron Database (Saxonov et al., 2000; Shepelev and Fedorov, 2006)) was used to receive the intron positions. At this time it offered the most up-to-date database, with available information about single species in separate datasets. Because some completely sequenced genomes were not included (for example *Oryza sativa*), the authors kindly provided their programs to generate a database of any genome sequence which is available in GenBank format. But using the EID raised some problems. Several human query sequences received from SWISS-PROT (Section 4.1) were not included in the database. So the analysis could not be performed without loosing a lot of information. Because of these problems a method was developed to obtain intron positions independently from annotations or database information, by taking only the sequence information into account.

4.2.2 A database independent method to identify intron positions

The database independent method to identify intron positions was implemented in several PERL scripts. Using BLAT (Kent, 2002), each query protein was aligned to its translated genomic region. Originally, BLAT was developed to align EST sequences to a complete reference genome in order to assemble and annotate a large amount of EST sequences in a moderate time frame. In contrast to BLAST the program BLAT aligns exons to the genome without overlapping ends of two consecutive hits. This is important and very useful for identifying the correct intron positions. If the gene is intronless, BLAT will find a hit in a consecutive nucleotide region. In Figure 4.2 two BLAT output files are shown as an example for this case. A protein from *Oryza sativa* and *Drosophila melanogaster* could be aligned over the total length of the protein with 100% sequence identity.

```
# BLAT 32 [2005/11/07]
# Query: osa_022056
# Database: /home/homes/nahal/Introns/Genomes_nt/osa/osa_list_homes
# Fields: Query id, Subject id, % identity, alignment length, mismatches, gap openings, q. start, q. end, s. start, s. end, e-value, bit score
osa_022056      osa_chr9        100.00  311     0       0       1       311     12242955        12242023        4.0e-186        647.0


# BLAT 32 [2005/11/07]
# Query: ath_008484
# Database: /home/homes/nahal/Introns/Genomes_nt/ath/ath_list_homes
# Fields: Query id, Subject id, % identity, alignment length, mismatches, gap openings, q. start, q. end, s. start, s. end, e-value, bit score
ath_008484      ath_chr2        100.00  125     0       0       1       125     3270407 3270781 2.4e-69 259.0
```

Figure 4.2: BLAT result for an intronless gene for a protein from *Oryza sativa* and *Arabidopsis thaliana*. The complete protein sequences are aligned to the genomic region with 100% identity.

The tabular BLAT output gives all information about the aligned regions, both of the protein and the nucleotide sequence. With this information the region of the nucleotide sequence can be extracted and analysed. In Figure 4.3 an example of another BLAT output is shown. In this example the *Drosophila melanogaster* query protein (dme_009528) has several hits on two different chromosomes (chr2R and chrX). Before checking for intron positions all BLAT output files were filtered to get the best aligned region on one chromosome and with all exonic regions. In the example of Figure 4.3(a), the hit on chromosome 2 (chr2) was chosen to be the best because the percent identity of all alignments is higher (all 100%

identity) and the total alignment length of 297 amino acids is larger than 186 amino acids on chromosome X. Figure 4.3(b) shows the BLAT output file after removing the hit of chromosome X which is also arranged by the positions of the query sequence. All BLAT results were filtered using these criteria described in this example.

```
# BLAT 32 [2005/11/07]
# Query: dme_008528
# Database: /home/homes/nahal/Introns/Genomes_nt/dme/dme_list_homes
# Fields: Query id, Subject id, % identity, alignment length, mismatches, gap openings, q. start, q. end, s. start, s. end, e-value, bit score
dme_008528  dme_chr2R  100.00  147  0  0  21  167  2695592 2696032 7.1e-84 308.0
dme_008528  dme_chr2R  100.00  130  0  0  168  297  2696089 2696478 6.5e-74 275.0
dme_008528  dme_chr2R  100.00  20  0  0  1  20  2695260 2695319 6.4e-02 35.0
dme_008528  dme_chrX  83.33  102  17  0  66  167  18789435  18789740  1.0e-46 184.0
dme_008528  dme_chrX  86.21  58  8  0  175  232  18789759  18789932  1.7e-25 114.0
dme_008528  dme_chrX  57.14  21  9  0  36  56  18789354  18789416  6.3e+00 29.0
dme_008528  dme_chrX  100.00  5  0  0  233  237  18820968  18820982  3.5e+06 10.0
```

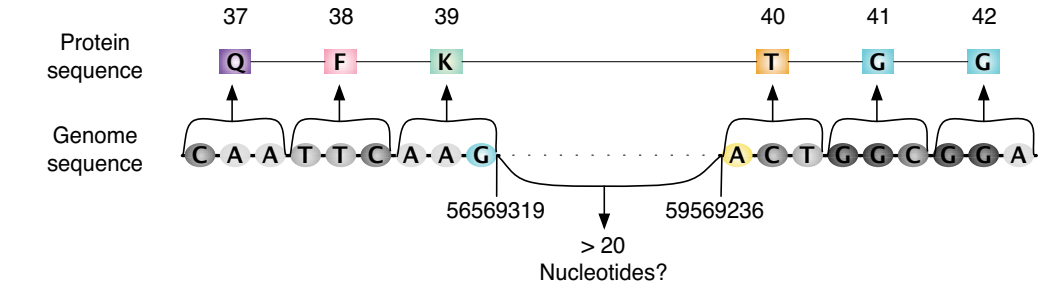
(a) BLAT result before filtering

```
# BLAT 32 [2005/11/07]
# Query: dme_008528
# Database: /home/homes/nahal/Introns/Genomes_nt/dme/dme_list_homes
# Fields: Query id, Subject id, % identity, alignment length, mismatches, gap openings, q. start, q. end, s. start, s. end, e-value, bit score
dme_008528  dme_chr2R  100.00  20  0  0  1  20  2695260 2695319 6.4e-02 35.0
dme_008528  dme_chr2R  100.00  147  0  0  21  167  2695592 2696032 7.1e-84 308.0
dme_008528  dme_chr2R  100.00  130  0  0  168  297  2696089 2696478 6.5e-74 275.0
```

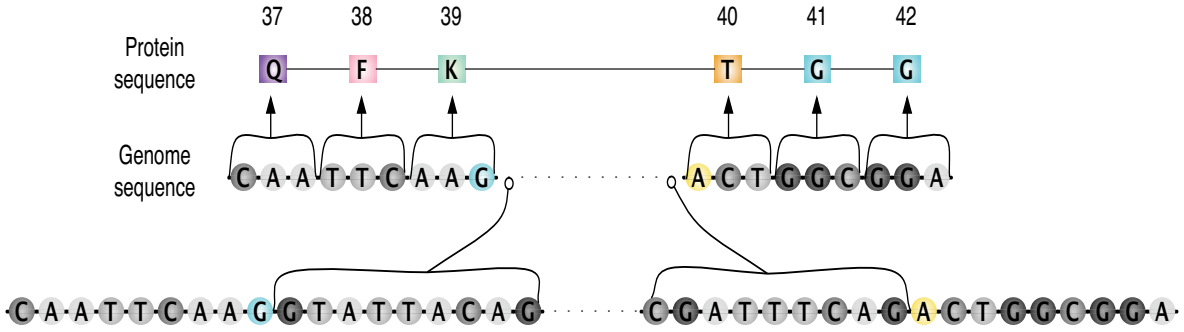
(b) BLAT result after filtering

Figure 4.3: BLAT hits of one protein found on two different locations on the genome of *Drosophila melanogaster*. Figure (a) shows the original BLAT output, figure (b) after filtering the best hit, sorted by the positions of the query sequence.

The procedure of testing if the region between two aligned exons is an intron or not is outlined in detail in Figure 4.4. A number of 20 nucleotides was defined as the minimum length of the region between two exons to be a probable intron which functions as a first step to identify an intron position (Figure 4.4(a)). To get the information about the length of this region, the difference of the given nucleotide positions is calculated. If the region is larger than 20 nucleotides, the second step in the method is to construct substrings. Each substring contains nine nucleotides of the coding region and nine nucleotides of the non-coding region of the probable intron sequence. These sequences were used for further analyses.



(a) First step



(b) Second step

Figure 4.4: First two steps of identifying intron positions at the sequence level. A part of an exon/intron boundary and the corresponding positions of the BLAT alignment are represented in each figure. To be designated a probable intron, the nucleotide region between the two hits must be larger than 20 nucleotides (a). The positions of the nucleotides in the genome are used to receive this information. Two substrings are created at each boundary consisting of at least nine nucleotides of the coding region and at least nine nucleotides in the noncoding region (b) which are used in the method further on.

In Figure 4.5 and Figure 4.6 two examples are shown to describe how the position and the phase of each intron were identified. The two substrings built in the second step of the procedure are checked for the canonical splicing sites GT and AG. In Figure 4.5 the BLAT alignment does not contain a gap within the query protein sequence. The sequence is cut at these positions and the two exon regions were joined as in the splicing procedure. The splicing sites have to be found in a relative position to each other so that in this region a frameshift does not appear. To test if the right splicing sites were found, the joined sequence is translated with the EMBOSS program transeq (Rice et al., 2000). With a string matching it is checked if the translated amino acid pattern is found in the original query protein at the site in question. If the pattern corresponds to the query

sequence, the position and the phase of the intron are identified. Figure 4.5 shows an example of the identification of an intron position of phase 0. The case is shown in Figure 4.6 if the query protein could not be aligned consecutively to the genome sequence. As mentioned before, the splicing sites must be found in a different distance, leading to an additional codon. After testing and identifying the intron positions, for each protein the intron positions and the corresponding phases are saved.

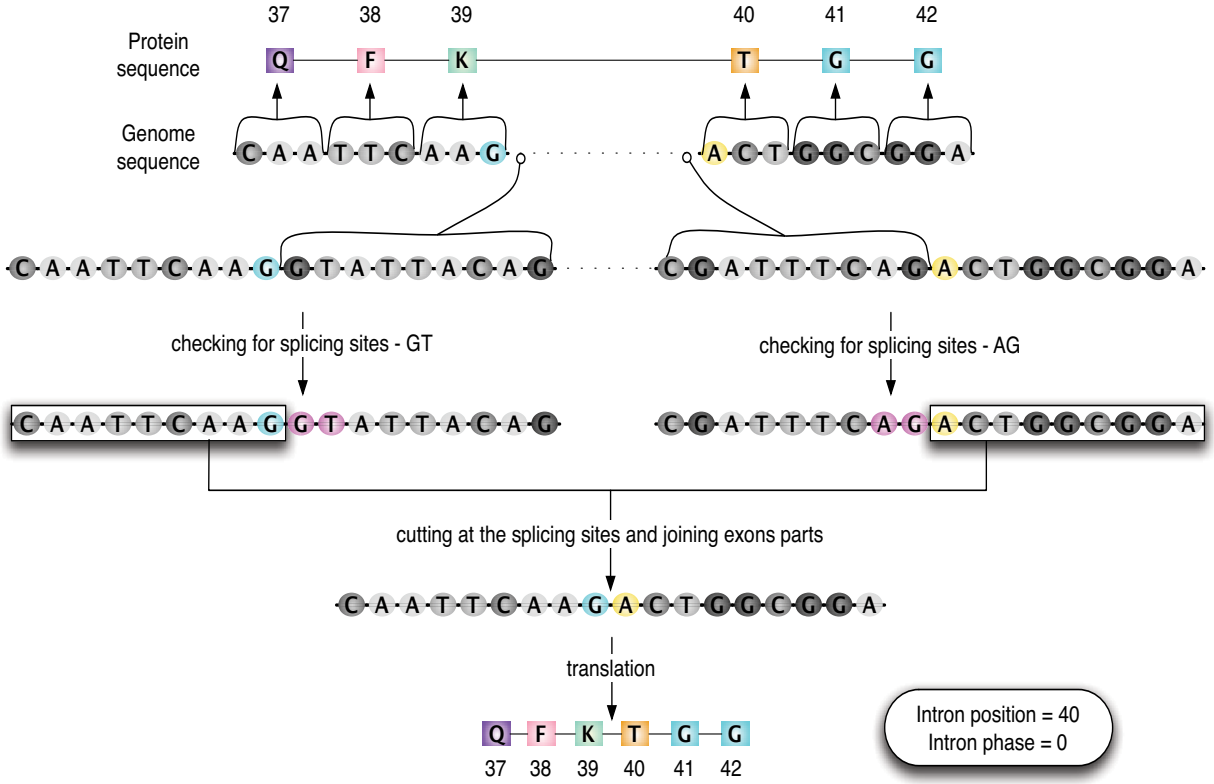


Figure 4.5: Identifying a phase 0 intron. The surroundig nucleotides are checked for canonical splicing sites. If the splicing sites exist, the two substrings are cut and rejoined as in the splicing procedure. The translation result in the query protein region, the intron position can be identified to be at position 40 in the protein and of phase 0.

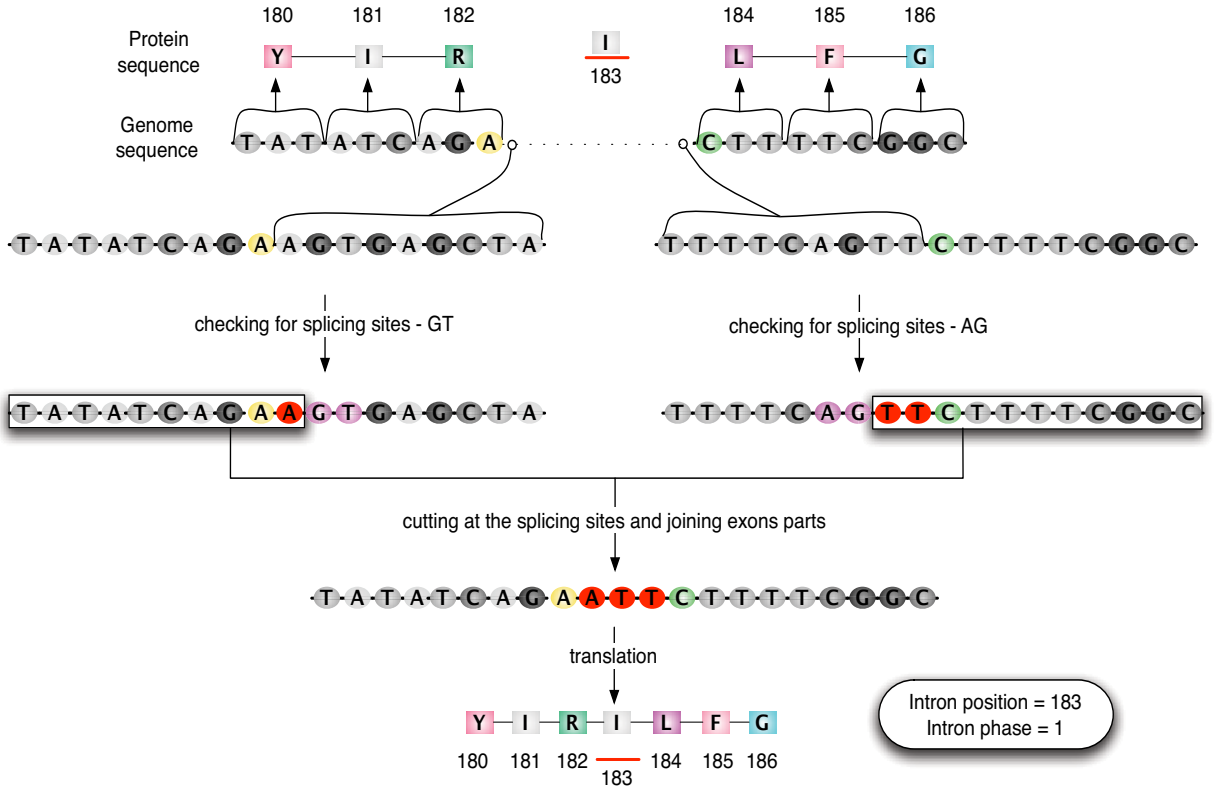


Figure 4.6: Identifying a phase 1 intron. Like shown in Figure 4.5, the same procedure is performed. The information of the not aligned amino acid at position 183 leads to a variation in the position of the splicing sites so that an additional codon arises. The translation results in the original protein sequence. In this case the intron lies at position 183 in the protein of phase 1.

The identification of a phase 2 intron follows the same procedure described for identifying phase 0 and phase 1 introns. For the automation of this method of intron identification, different variations in the data must have been considered. The BLAT hit could be found in the reversed or the complement reversed genome sequence, which has to be considered in the implementation of the extraction of the corresponding nucleotide region out of the genome sequence. Other possible cases that could occur in the data were marked during these procedures and checked manually. The different cases were the following (a) the splicing sites are found too distant from each other, (b) the nucleotide region is smaller than 20 nucleotides, (c) no splicing sites are found, (d) the translation of the exon parts does not match the query protein, (e) the hits of the query sequence are overlapping.

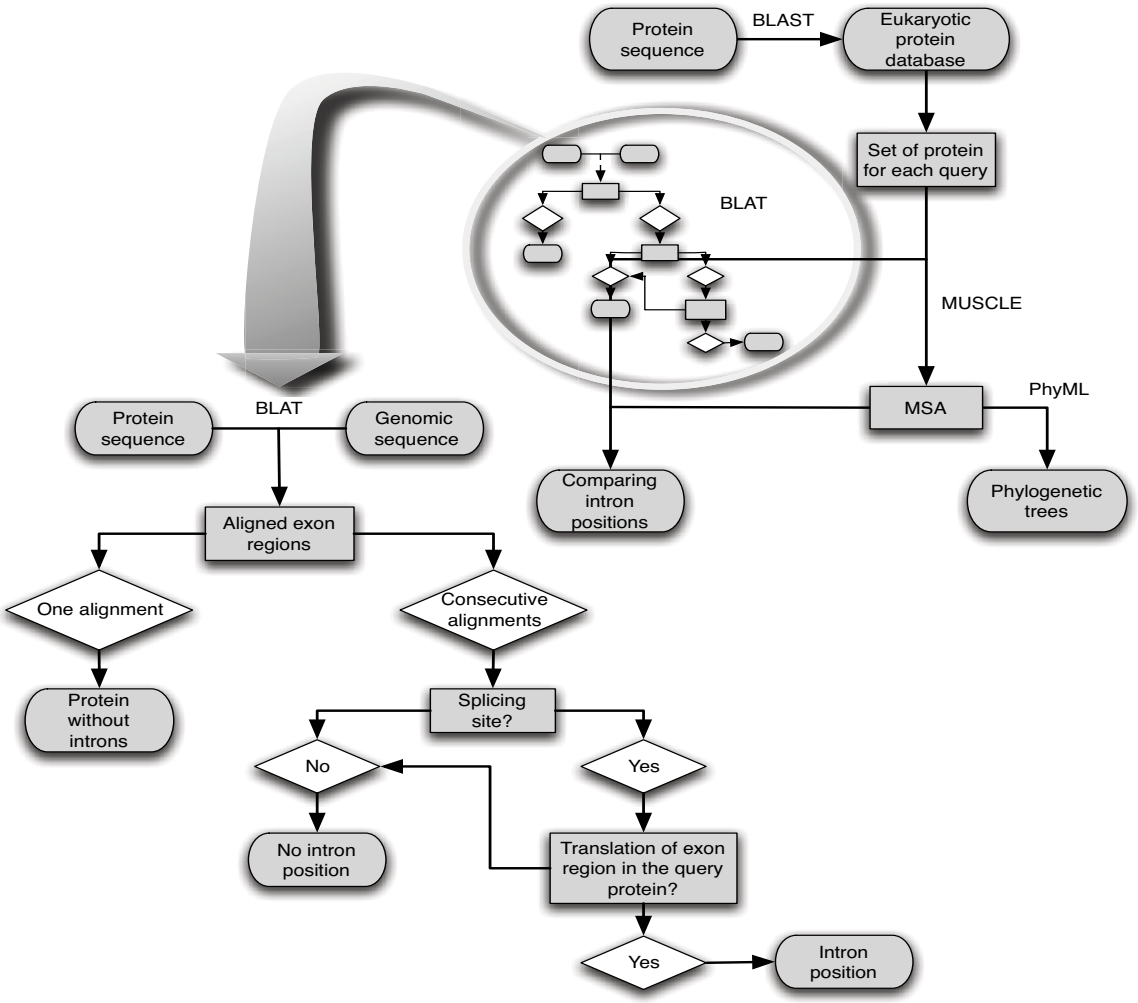


Figure 4.7: General workflow to summarize the main steps described in this section.

4.2.3 Comparison of intron positions

The multiple sequence alignments computed with MUSCLE (Edgar, 2004b,a) (Section 4.1.2) were used to compare the intron positions within the homologous sequences. All procedures described in the following were implemented in perl[2]-scripts. The identified intron positions of each protein were rearranged to their relative position in the alignment. Therefore the number of gap sites in one sequence preceding an intron position was added to the following positions. The fasta format was chosen as the output file format of MUSCLE in

[2]http://www.perl.org/

which all sequences of the alignment are sequentially arranged in one file, containing the gap positions. For each alignment, a presence/absence matrix of all intron positions was constructed (Figure 4.8(a)) from which a 1-0 profile for each group of organisms was built (Figure 4.8(b)). The group animals contains the species *Caenorhabditis elegans*, *Danio rerio*, *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*. The plant group comprises the three species *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, and *Oryza sativa*. The five species *Aspergillus fumigatus*, *Candida glabrata*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica* constitute the fungi group. Each of the other four eukaryotes build their own single group, *Dictyostelium discoideum*, *Leishmania major*, *Plasmodium falciparum*, and *Thalassiosira pseudonana*.

yli_005238	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
afu_000647	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
ddi_002209	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
cel_009028	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
dre_008019	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0
dre_013251	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0
hsa_000217	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0
rno_010183	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0
rno_007791	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0
mmu_037423	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0
cre_007107	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	1
dme_005550	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
tps_000052	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
osa_009645	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1
ath_007611	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1
ath_001893	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1

(a) Presence/absence matrix of intron positions

Animals	1	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	0
Plants	0	1	1	0	0	1	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0	1
Fungi	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
D.discoideum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
T.pseudonana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

(b) Group profile of intron positions

Figure 4.8: Presence/absence matrix and group profiles of intron positions. For each sequences in a multiple sequence alignment, the presence (1) or absence (0) of an intron among all intron positions among all sequences is indicated in a matrix (a). The different groups of organisms are color-coded (blue = animals, green = plants, grey = fungi, yellow = *Dictyostelium discoideum*, red = *Thalassiosira pseudonana*). The matrix in (b) represent the merged 0-1 profiles for each group.

Shared intron positions were identified using these profiles, for the whole align-

ment and only for conserved alignment regions, determined with Blockmaker[3] (Henikoff et al., 1995), a feature at the Blocks database (Henikoff and Henikoff, 1991).

4.2.4 Intron density and intron phases

For a single gene, the intron density was calculated as the number of introns per kilo basepairs of the coding sequence. This number is independent of the gene-length. The intron density for one species, encompassing more than one gene is represented by the mean intron density for the genes under consideration. The intron phases are given as percentages so that the distributions of intron phases represent the fractions of introns in each phase of the total number of introns in the corresponding genes.

4.3 Timing of endosymbiotic gene transfer

Using the information of presence/absence of the mitochondrial and proto-mitochondrial genes in the genomes of the different species, the relative time of endosymbiotic gene transfer events could be defined.

A table was constructed, containing the information about the presence in the mitochondrial genome. The information was taken from the protein tables of the mitochondrial genomes of NCBI. In Table 4.9 the RefSeq accession numbers are listed of the mitochondrial genomes of each species. This information was not available for *Leishmania major* and *Aspergillus fumigatus*, which were replaced by the species *Leishmania tarentolae* and *Aspergillus niger*, respectively. The number of protein coding genes in the mitochondrial genomes is also listed in Table 4.9.

[3] http://blocks.fhcrc.org/blocks/

Table 4.9: RefSeq accession numbers of the mitochondrial genomes in NCBI for all eukaryotes under consideration. Each RefSeq mitochondrial (mt) genome contains a protein table, from which the gene content in the mitochondria of the different species was detected. In the last column, the number of protein coding genes is presented.

Organism (mt)	RefSeq accession	# protein coding genes
Arabidopsis thaliana	NC_001284	117
Chlamydomonas reinhardtii	NC_001638	8
Oryza sativa	NC_011033	53
Dictyostelium discoideum	NC_000895	42
Leishmania tarentolae	NC_000894	2
Plasmodium falciparum	NC_002375	3
Thalassiosira pseudonana	NC_007405	35
Aspergillus niger	NC_007445	16
Candida glabrata	NC_004691	11
Saccharomyces cerevisiae	NC_001224	19
Schizosaccharomyces pombe	NC_001326	10
Yarrowia lipolytica	NC_002659	24
Danio rerio	NC_002333	13
Drosophila melanogaster	NC_001709	13
Caenorhabditis elegans	NC_001328	12
Homo sapiens	AC_000021	13
Mus musculus	NC_010339	13
Rattus norvegicus	AC_000022	13

The time of endosymbiotic gene transfer of mitochondrial genes during evolution of the different eukaryotes was specified within the taxonomic relationship between the species and the different gene contents of the mitochondria. The taxonomic relationships between the species are reresented in a phylogenetic tree, in accordance to the phylogenies reported in two independent publications (Sugden et al., 2003; Keeling et al., 2005). The time of each endosymbiotic gene transfer event is inferred in a parsimonious way in which a transfer is labelled at the deepest node as possible in the phylogenetic tree to explain the existence of the gene in the nucleus of the species with a minimum number of transfer events.

4.4 Phylogenetic analyses and data visualization

4.4.1 Multiple alignments with intron positions

As described in Section 4.2.3, intron positions were rearranged in the multiple sequence alignments to compare the positions of the introns. For counting the number of species specific and shared intron positions, the presence/absence matrix of intron positions is useful for an automated process but for a closer examination of individual cases, a graphical representation is much more comfortable. For this reason, the intron positions were graphically mapped onto the alignments. Using HTML (Hypertext Markup Language), the intron positions and the group of species were color-coded in the alignment files which could than be opened reviewed in a web browser. Additionally, the species were sorted and the conserved alignment regions inferred with Blockmaker (Section 4.2.3) were highlighted.

4.4.2 Phylogenetic trees and median networks

Maximum likelihood trees were computed with the program phyml (Guindon and Gascuel, 2003) for all homologous sets of the human query sequences. Therefore the alignments were reformatted from the clustal format into the phylip format, which is a required input file format. Before reconstructing the phylogenetic trees, all gap positions in the alignments were removed. The graphical display of the trees was done with the program FigTree[4]. This program can read the output files of the program phyml in newick format.

The binary information of the presence/absence patterns of intron positions in the alignments were used to reconstruct median networks (Bandelt et al., 1995, 2000). The computation and visualization was performed with the program SplitsTree (Huson, 1998).

4.4.3 Comprehensive phylogeny of the *nad7* gene

To resolve the phylogeny of the gene *nad7* in more detail, the mitochondrial encoded *nad7* genes were added from the two plants *Arabidopsis thaliana* and

[4]http://tree.bio.ed.ac.uk/software/figtree/

Oryza sativa, the moss *Physcomitrella patens*, the two green algae *Pseudendoclonium akinetum* and *Ostreococcus tauri*, the diatom *Thalassiosira pseudonana*, and the slime mold *Dictyostelium discoideum*. The nuclear encoded *nad7* gene in the green alga *Volvox carteri* was added, too. All these *nad7* protein sequences were aligned with MUSCLE (Edgar, 2004b,a), and the gapped sites were removed. Because the *nad7* data sample includes eukaryotic nuclear sequence, mitochondrial sequences, and prokaryotic sequences, the phylogenetic reconstruction method has to take different evolutionary rates into account (Bruno and Halpern, 1999; Singer and Hickey, 2000). Therefore the program ProtTest (Abascal et al., 2005) was used to estimate which substitution model fits the data best. ProtTest computes maximum likelihood trees using phyml (Guindon and Gascuel, 2003) under different substitution models and outputs the most likely tree according to different criteria. The maximum likelihood values for the trees are then used to perform a goodness of fit test. The AICc (Akaike Information Criterion with a second order correction for small sample sizes) (Akaike, 1973) and the BIC (Bayesian Information Criterion) were used. In all cases the WAG (Whelan and Goldman, 2001) substitution model with an estimated proportion of invariable sites and a Γ-distribution (WAG+I+G) was chosen to explain the evolution of *nad7* best. Bootstrap values were calculated using this model with 100 bootstrap replicates. The phylogenetic tree is rooted by a clade of α-proteobacterial *nad7* genes.

The codon usage of the amino acid Glutamine (Q) was compared between the mitochondrial and the nuclear genomes which were included in this tree. The percentage of used codons was received from the codon usage database[5] (Nakamura et al., 2000) for each single species and averaged for the mitochondrial and the nuclear genomes, respectively.

[5]http://www.kazusa.or.jp/codon/

4.5 Survey

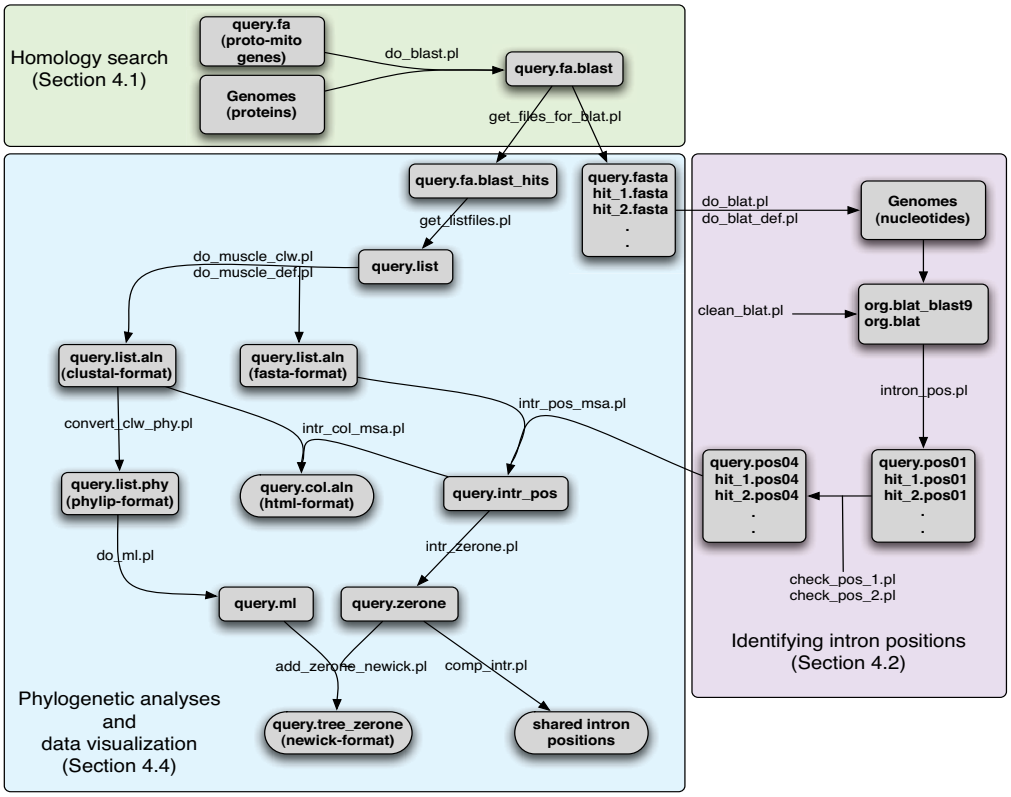


Figure 4.9: Survey of the workflow framework including detailed information about the realization of the methods used in this work. The gray boxes represent the input and output files of each steps, including the format of the output files in parenthesis. The .pl files are specifically programmed perl scripts which perform each task. The colour highlighted parts represents the corresponding sections.

Figure 4.9 gives a graphical survey of the workflow framework of the described method in this section. The .pl files are the specifically programmed perl scripts that were used to perform the different processes, pointing out to the corresponding section in the coulour highlighted boxes. The gray boxes represent the input/output files in which general file formats are indicated.

5 Results

5.1 Database and annotation independent method to identify intron positions

A dataset containing phylogenetically diverse species, representing the range of eukaryotes with completely sequenced genomes to date was crucial for the study of the evolution of spliceosomal introns but the use of existing databases of intron/exon structure would have limited the number of available species and genes in the analysis. Therefore, the development of a method to identify intron positions without using genome annotation data was necessary to be able to to include the 18 eukaryotic genomes (Section 4.2.2). In the complete dataset of 1861 proteins, 5099 intron positions were identified. The numbers of idetified intron positions for all five protein complexes of the oxidative phosphorylation pathway and the large and the small subunit of the mitochondrial ribosome are listed in Table 5.1.

Table 5.1: Number of identified intron positions (a) and number of proteins (b) of the oxidative phosphorylation pathway (Comp I - Comp V) and proteins of the large (L) and small (S) mitochochondrial ribosome subunit.

(a) Number of identified intron positions

	Comp I	Comp II	Comp III	Comp IV	Comp V	L	S	total
	1185	292	717	348	1107	997	453	
total	3649					1450		5099

(b) Number of proteins

	Comp I	Comp II	Comp III	Comp IV	Comp V	L	S	total
	445	93	199	200	351	388	185	
total	1188					573		1861

On average, the genes in this dataset contain 2.74 introns per gene (Table 5.1). From the manually checked cases (Section 4.2.2), 123 probable intron positions

were excluded from the analyses. In most of these (70 cases) introns were not validated because no canonical splicing sites were found (Table 5.2).

Table 5.2: Number of excluded intron positions in proteins of the oxidative phosphorylation pathway (Comp I - Comp V) and proteins of the large (L) and small (S) mitochochondrial ribosome subunits.

	Comp I	Comp II	Comp III	Comp IV	Comp V	L	S	total
too distant	9	0	4	2	2	3	4	24
too short	2	0	1	1	3	3	3	13
no sp	11	7	9	6	13	23	1	70
no trans	0	0	0	1	3	8	2	14
overlapping	0	0	0	0	1	1	0	2
total	22	7	14	10	22	38	10	123

too distant - the splicing sites are too distant from each other
too short - the putative intron is shorter than 20 nucleotides
no sp - no splicing sites are found
no trans - translation of the exons does not match the query protein
overlapping - the hits of the query sequence are overlapping

Non-canonical introns were not considered in this analysis. Because of the weakly conserved pattern of their splicing sites ([A/G]T-A[C/G]), the identification is not as clear as in the cases of canonical splicing sites. Spliceosomal introns with canonical splice sites account for more than 99.5% of introns in all eukaryotes (Rodríguez-Trelles et al., 2006). Additionally, because of the fact that some species do not even contain non-canonical introns in their genomes, as for example *Caenorhabditis elegans* (Burge et al., 1998), the analysis was focussed on canonical introns. It is known, that non-canonical splicing sites can change into canonical splicing sites (GT-AG) while still being spliced by the minor spliceosome (Burge et al., 1998; Will and Luhrmann, 2005). In these cases, the introns were treated as canonical introns.

The information about intron positions was stored in single files for each protein sequence with the corresponding intron phases (Figure 4.9, ".pos04" files). These files provided the basis for further steps in the analyses.

5.2 Proteins of the oxidative phosphorylation pathway

The proto-mitochondrial genes of the oxidative phosphorylation pathway are listed in Table 5.3. All these genes have a homologous sequence in the α-proteobacterium *Rickettsia prowazekii* and are referred to as proto-mitochondrial genes. If the corresponding mitochondrial gene name could be derived by sequence comparison with the mitochondrial genome of *Reclinomonas americana* (Section 4.1.1), the name is listed in the table.

Table 5.3: Proto-mitochondrial genes of the oxidative phosphorylation pathway in *Homo sapiens* are listed with their SWISS-PROT identifier and their corresponding mitochondrial gene names, if possible.

Complex I	
SWISS-PROT ID	mitochondrial gene name
O75306	*nad7*
O00217	*nad8*
O75489	*nad9*
O75251	*nad10*
P28331	*nad11*
P19404	-
P49821	-
Complex II	
SWISS-PROT ID	mitochondrial gene name
P21912	*sdh1*
P31040	*sdh2*
Q99643	*sdh3*

Complex III	
SWISS-PROT ID	mitochondrial gene name
P08574	-
P47985	-
Complex IV	
SWISS-PROT ID	mitochondrial gene name
P99999	-
Complex V	
SWISS-PROT ID	mitochondrial gene name
P06576	*atp1*
P25705	*atp1*
P36542	*atp3*
P48201	*atp9*
Q06055	*atp9*
P05496	*atp9*
P48047	-

For all genes of Table 5.3, the genomic location is listed in Table 5.4. In addition to the mitochondrial gene content information in the different species, Table 5.4 indicates, which sequences could be identified with the homology search with the human query sequences as described in Section 4.1.2. If a gene is found by homology search but is not encoded in the mitochondrial genome, it can either have been lost or it was not possible to identify it under the homology search restrictions used here (*Homo sapiens* as a query, e-value threshold), or it is not present in the protein data set.

Table 5.4: Genomic location of genes of the oxidative phosphorylation pathway.

Human Swiss-Prot ID	mitochondrial gene name	*Homo sapiens*	*Drosophila melanogaster*	*Danio rerio*	*Caenorhabditis elegans*	*Mus musculus*	*Rattus norvegicus*	*Aspergillus fumigatus*	*Yarrowia lipolytica*	*Candida glabrata*	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Chlamydomonas reinhardtii*	*Arabidopsis thaliana*	*Oryza sativa*	*Dictyostelium discoideum*	*Leishmania major*	*Plasmodium falciparum*	*Thalassiosira pseudonana*
O75306	*nad7*	N	N	N	N	N	N	N	N	-	L	L	N	M	M	M	M	L	M
O00217	*nad8*	N	N	N	N	N	N	N	N	-	L	L	N	N	N	N	-	L	N
O75489	*nad9*	N	N	N	N	N	N	N	N	-	L	L	N	M	M	M	-	L	M
O75251	*nad10*	N	N	N	N	N	N	N	N	-	L	L	N	N	N	N	N	L	N
P28331	*nad11*	N	N	N	N	N	N	N	N	-	L	L	N	N	N	-	N	L	M
P19404		N	N	-	N	N	N	N	N	-	-	N	N	N	N	N	N	-	N
P49821		N	N	N	N	N	N	N	N	-	-	N	N	N	N	N	N	-	N
P21912	*sdh1*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
P31040	*sdh2*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Q99643	*sdh3*	N	N	N	N	N	N	N	N	N	-	N	-	-	-	N	-	-	N
P08574		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
P47985		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
P99999		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
P06576																			
P25705	*atp1*	N	N	N	N	N	N	N	N	N	N	N	N	M&N	M	M	N	N&L	N
P36542	*atp3*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N&L	N
P48201																			
Q06055	*atp9*	N	N	N	N	N	N	M&N	M	M	M	M	N	M&N	M	M	N	L	M
P05496																			
P48047		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	N	N

N – the gene is located in the nuclear genome of the species
M – the gene is located in the mitochondrial genome
L – the gene is lost in the species
'-' – the gene could not be identified with the homology search

Most of the mitochondrial encoded genes are found in the four species *Arabidopsis thaliana*, *Oryza sativa*, *Dictyostelium discoideum* and *Thalassiosira pseudonana*. Only proven cases of gene loss are indicated in the table ("L", Table 5.4). The complete loss of Complex I, and thus the loss of the corresponding genes, is reported for the two yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Gabaldón et al., 2005) and in *Plasmodium falciparum* which also lacks the ATPase genes (Gardner et al., 2002). Surprisingly, the two genes *atp1* and *atp3* were identified in the genome of *Plasmodium falciparum* (NP_701707 and NP_705013, respectively). A recent reevaluation revealed that the entries of these two nuclear encoded genes were removed from the actual version of the database NCBI and therefore subsequently excluded from this analysis. The gene *atp1* in *Arabidopsis thaliana* and the gene *atp9* in *Arabidopsis thaliana* and *Aspergillus fumigatus* appear to be encoded in both, the mitochondrial and the nuclear genome. These cases were studied in detail with the results summarized in Table 5.5

Table 5.5: Genes of the oxidative phosphorylation pathway encoded in the mitochondrial and the nuclear genome. The accession numbers are given for each gene and the definitions annotated in NCBI. The chromosomal location of the nuclear encoded gene and the pairwise identities between the mitochondrial and the nuclear genes, computed by BLAST are listed.

genomic location	Accession number (gene description)	pairwise identity
atp1 - Arabidopsis thaliana		
nucleus (chromosome 2)	NP_178788 (ATP synthase α chain, mitochondrial, putative)	99%
mitochondrion	NP_085571 (ATPase subunit 1)	
atp9 - Arabidopsis thaliana		
nucleus (chromosome 2)	NP_178769 (H+-transporting two-sector ATPase, C subunit family protein)	100%
mitochondrion	NP_085561 (ATPase subunit 9)	
atp9 - Aspergillus fumigatus		
nucleus (chromosome 4)	XP_751890 (ATP synthase subunit ATP9)	67%
mitochondrion (*Aspergillus niger*)	YP_337886 (ATP synthase subunit 9)	

The genes *atp1* and *atp9* were clearly identified on a chromosomal location in the genomes of *Arabidopsis thaliana* and *Aspergillus fumigatus*, respectively. A pairwise sequence comparison between the nuclear and the mitochondrial gene of the corresponding species was calculated with BLAST and resulted in 99% and 100% identity in *Arabidopsis thaliana* and in 67% identity in *Aspergillus fumigatus*. It is important to mention that the mitochondrial genome of *Aspergillus fumigatus* is not sequenced yet and that the information on mitochondrial gene content was replaced with that of *Aspergillus niger* (Section 4.3). With the nuclear encoded gene *atp9* of *Aspergillus fumigatus* a BLAST search was performed in the nuclear genome of *Aspergillus niger*. The two best BLAST hits with e-values of 2e-58 and 4e-58 were annotated as "hypothetical protein" (XP_001402177) and

"ATP synthase 9" (1510195A), respectively. That would suggest the existence of *atp9* in the nuclear genomes of the two fungi. A third significant hit of this BLAST search with an e-value of 3e-21 was the mitochondrial encoded gene of *Aspergillus niger* (YP_337886).

The endosymbiotic gene transfer events of genes of the oxidative phosphorylation pathway were mapped onto the branches of a tree which represents the taxonomic relationships between the species (Figure 5.1). Depending on the presence or absence of a gene in the mitochondrial genomes, the parsimonious inference of gene transfer events result in this picture of endosymbiotic gene transfer among the evolution of these species. Each transfer is labelled at the earliest possible speciation event. For example, the genes *nad8*, *nad10*, *atp3*, and *sdh2,3,4* are transferred at an early stage of eukaryotic evolution because they are nuclear encoded in all species in this tree. In the group of plants and green algae, there are nine genes which were only transferred to the nuclear genome in *Chlamydomonas reinhardtii*. The timing of transfer events does not exclude occurrences of gene losses, but the labelled genes are not present in the mitochondrial genomes of the corresponding species. There are no independent transfer events within the groups of vertebrates and fungi which are both represented in the tree in Figure 5.1 as a single clade each.

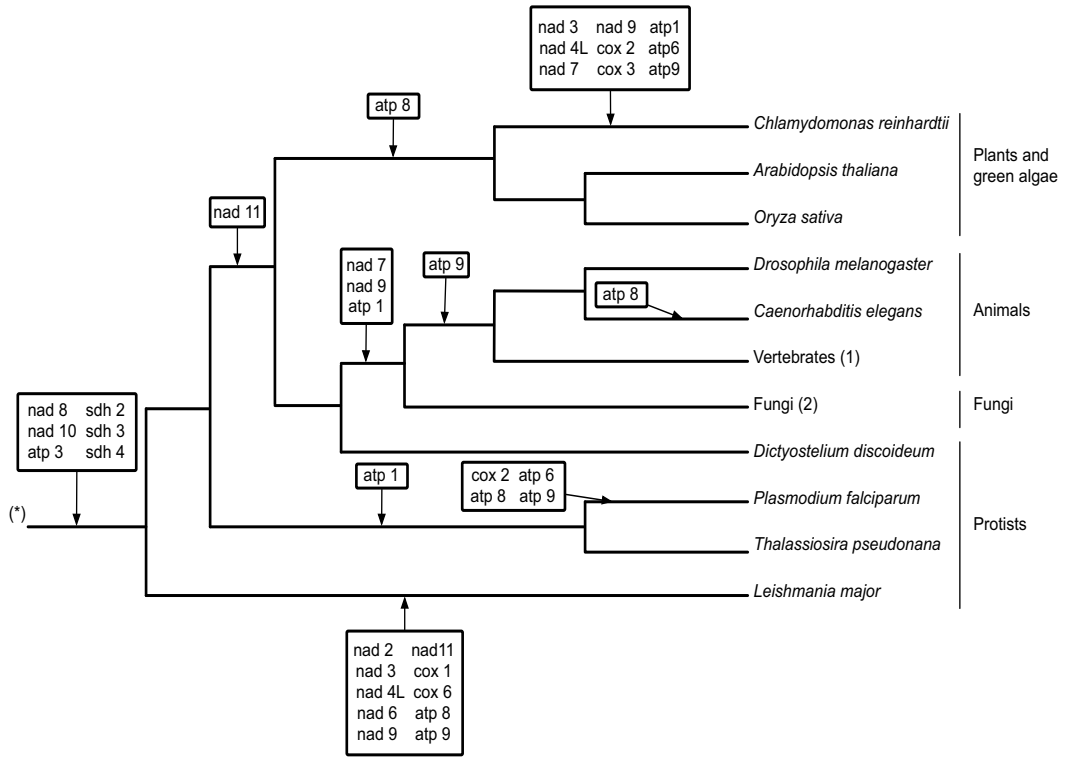
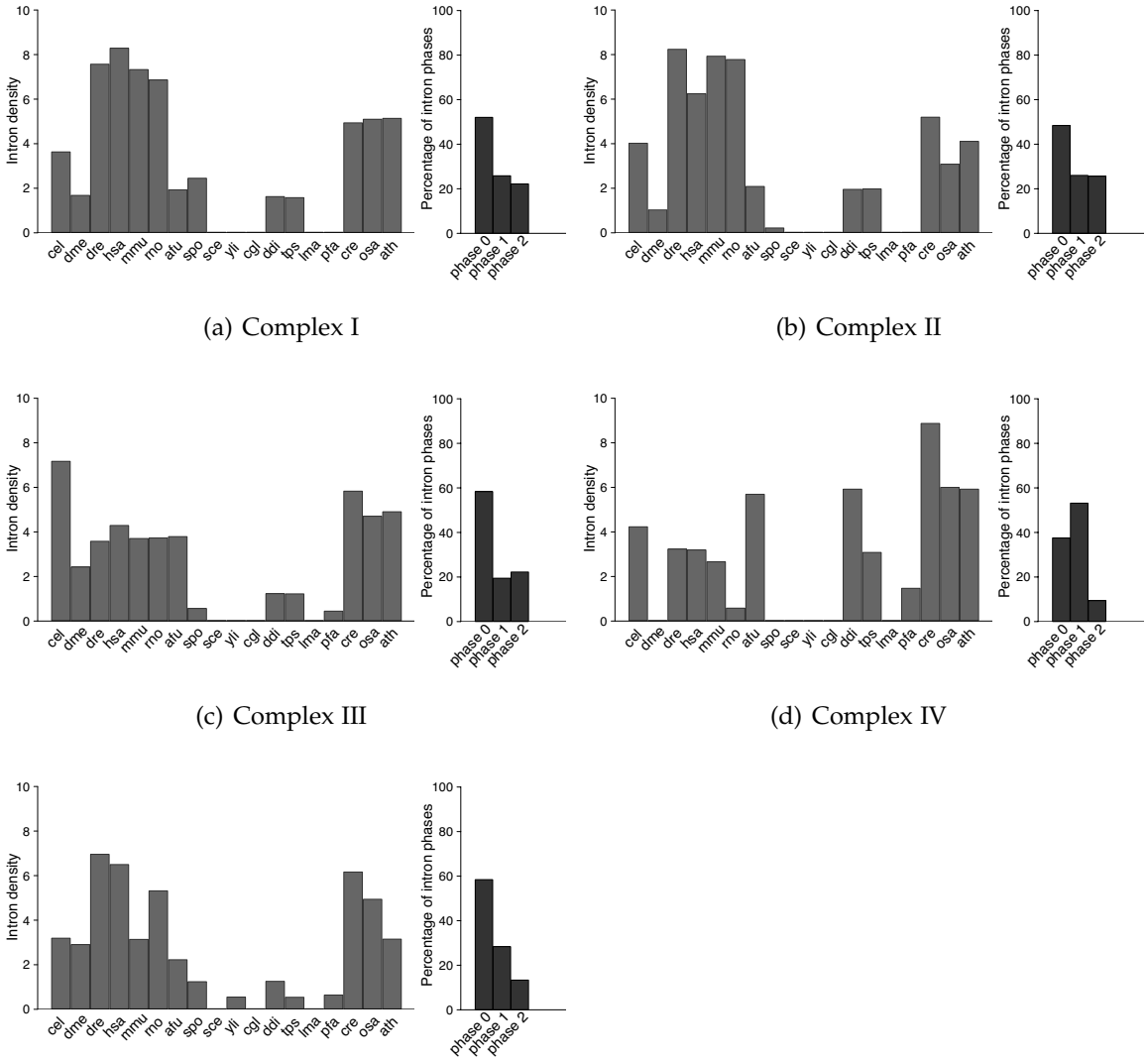


Figure 5.1: Timing of endosymbiotic gene transfer of proto-mitochondrial genes of the oxidative phosphorylation pathway. Gene transfer events are labelled at the branches. The tree represents the taxonomic relationships between the species. The time of transfer of a gene is relative to the speciation events depending on the presence/absence in the mitochondrial genome (Section 4.3). (1) - *Danio rerio, Homo sapiens, Mus musculus, Rattus norvegicus* (2) - *Aspergillus fumigatus, Schizosaccharomyces pombe, Yarrowia lipolytica, Saccharomyces cerevisiae, Candida glabrata*. (*) - bacteria and archaea.

5.2.1 Intron densities and phase distributions

The intron densities and the distributions of intron phases in proto-mitochondrial genes of the oxidative phosphorylation pathway are shown in Figure 5.2 for each species. The highest intron densities are found in the groups of animals and plants. Especially *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, and *Oryza sativa* show constant densities in all different complexes.



(a) Complex I

(b) Complex II

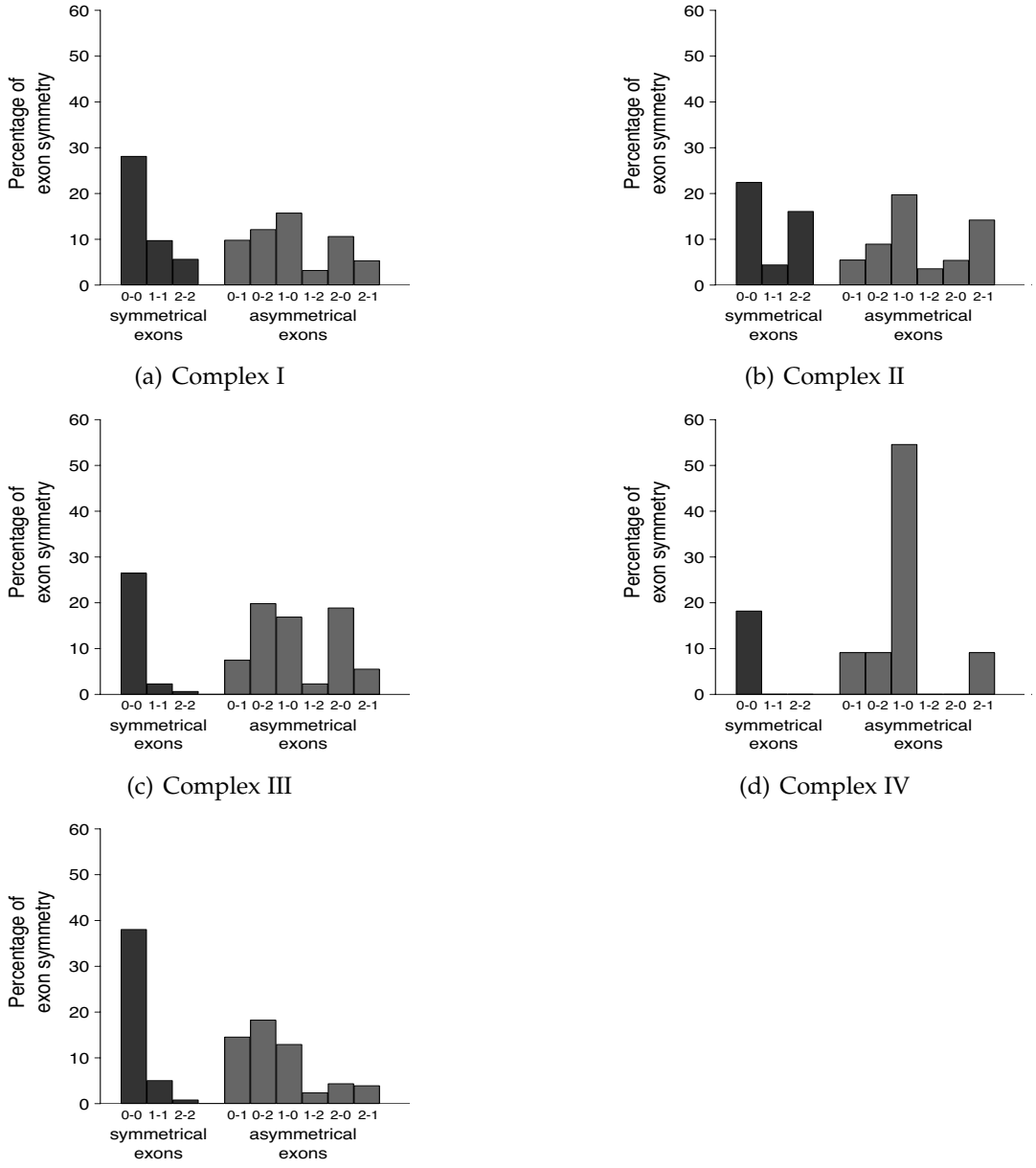
(c) Complex III

(d) Complex IV

(e) Complex V

Figure 5.2: Intron densities and phase distributions in genes of the oxidative phosphorylation pathway. Intron densities for each species in all different protein complexes of the oxidative phosphorylation pathway are shown with the percentages of intron phases. Intron densitiy is given as the number of introns per kilo basepairs of coding sequence. cel=*Caenorhabditis elegans*, dme=*Drosophila melanogaster*, dre=*Danio rerio*, hsa=*Homo sapiens*, mmu=*Mus musculus*, rno=*Rattus norvegicus*, afu=*Aspergillus fumigatus*, spo=*Schizosaccharomyces pombe*, sce=*Saccharomyces cerevisiae*, yli=*Yarrowia lipolytica*, cgl=*Candida glabrata*, ddi=*Dictyostelium discoideum*, tps=*Thalassiosira pseudonana*, lma=*Leishmania major*, pfa=*Plasmodium falciparum*, cre=*Chlamydomonas reinhardtii*, osa=*Oryza sativa*, ath=*Arabidopsis thaliana*.

Among all species analyzed, the highest intron density is found in the green alga *Chlamydomonas reinhardtii* with 6.17 introns per kilo basepairs (kb) coding seqence, followed with 5.89 and 5.68 introns per kb coding sequence in *Danio rerio* and *Homo sapiens*, respectively. The genes of the three species *Candida glabrata*, *Saccharomyces pombe*, and *Leishmania major* do not contain introns. Complex IV (Figure 5.2(d)) shows a differing intron density and differing distribution of intron phases in comparison to the other protein complexes. In complex IV, the highest number of introns is of phase 0, whereas in all other cases the highest percentages are those of phase 0 introns. The summed up percentages of introns of phase 0 (50.93%), phase 1 (30.54%), and phase 2 (18.54%) result in a ratio of 5:3:2.



(a) Complex I

(b) Complex II

(c) Complex III

(d) Complex IV

(e) Complex V

Figure 5.3: Exon symmetry distribution in genes of the oxidative phosphorylation pathway. The percentage of symmetrical and asymmetrical exons is shown separately for the five different Complexes I-V.

The distribution of symmetrical and asymmetrical exons in the genes of oxidative phosphorylation is shown in Figure 5.3. In total, with 26.62% the 0-0 exons account for the highest amount of symmetrical exons in all protein complexes.

The asymmetrical 1-2 exons form the smallest group with only 2.26%. Only in complex IV, Figure 5.3(d), the amount of 1-0 asymmetrical exons (54.55%) exceeds the number of 0-0 exons (18.18%).

Intron densities of several selected genes were examined with regard to the timing of endosymbiotic gene transfer events (Figure 5.1). These genes represents transfer events at different evolutionary stages in the evolution of the four eukaryotes, *Homo sapiens*, *Danio rerio*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Figure 5.4). The two early transfers *nad8* and *nad10* are followed by the genes *nad11* and four other genes *nad7*, *nad11*, *atp1*, and *atp9*, the latter representing the most recent gene transfer. The highest intron density among all genes is found in the two species *Danio rerio* and *Homo sapiens*. A lower intron density is found in the genes of the other two species *Drosophila melanogaster* and *Caenorhabditis elegans*. A slightly higher intron density can be observed in the anciently transferred genes *nad8* and *nad10* of *Caenorhabditis elegans* and in the gene *nad8* of *Drosophila melanogaster*. There is no correlation between intron density and the time of gene transfer.

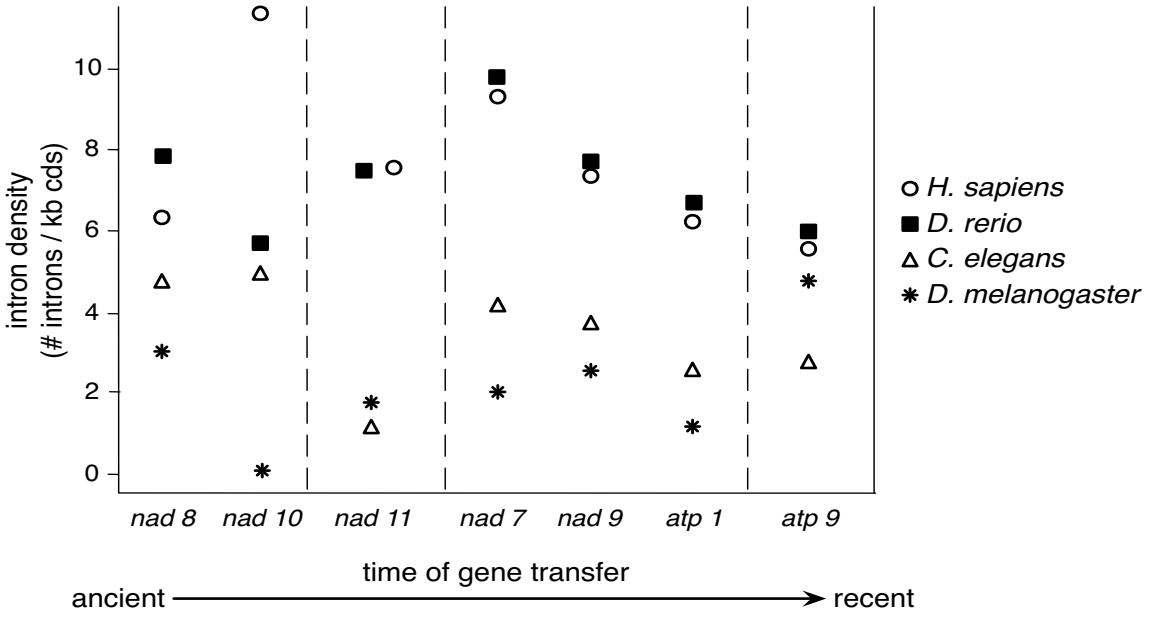


Figure 5.4: Intron density in genes transferred at different evolutionary stages in *Homo sapiens*, *Danio rerio*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. Four different evolutionary times of endosymbiotic gene transfer are represented, with the two genes *nad8* and *nad10* as the most ancient gene transfers and the gene *atp9* as the most recent gene transfer in all four eukaryotes.

5.2.2 Shared intron positions

Among the multiple sequence alignments, the number of shared intron positions between the different groups of species were computed. Table 5.6 presents the species specific intron positions in the groups of animals, plants, fungi, and the other species in the diagonal. The table contains both, shared intron positions among the complete alignments and only in conserved regions of the alignments, determined with Blockmaker (Section 4.2.3) above and below the diagonal, respectively.

Table 5.6: Shared intron positions in genes of the oxidative phosphorylation pathway. Shared intron positions between the different groups of species within the complete multiple protein alignments are shown above the diagonal, shared intron positions only within conserved regions of the alignment are shown below the diagonal. Species specific intron positions are shown in the diagonal.

	Animals	Plants	Fungi	D. dis	L.maj	P. fal	T. pse
Animals	**287**	60	12	4	-	-	1
Plants	4	**285**	1	-	-	-	3
Fungi	1	0	**78**	-	-	-	-
D. dis	1	-	-	**15**	-	1	-
L. maj	-	-	-	-	**0**	-	-
P. fal	-	-	-	0	-	**7**	-
T. pse	0	0	-	-	-	-	**24**

Animals - *Caenorhabditis elegans, Danio rerio, Drosophila melanogaster, Homo sapiens, Mus musculus, Rattus norvegicus*
Plants - *Arabidopsis thaliana, Chlamydomonas reinhardtii, Oryza sativa*
Fungi - *Aspergillus fumigatus, Candida glabrata, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Yarrowia lipolytica*
D. dis - *Dictyostelium discoideum*
L.maj - *Leishmania major*
P. fal - *Plasmodium falciparum*
T. pse - *Thalassiosira pseudonana*

The number of shared intron positions in conserved alignment regions is highly reduced compared to shared positions in the complete alignments, but still shows the same trend. Most intron positions are shared between the most divergent groups animals and plants, with 73,2 % out of 82 shared intron positions among the complete alignment lengths, and 66,7 % out of six shared intron positions identified in the conserved alignment regions.

Only few intron positions are found across more than two groups of organisms. Altogether 12 intron positions are shared between three groups, whith

seven of them found in animals, fungi and plants, and two positions shared between animals, plants and *Dictyostelium discoideum* and one shared intron position between animals, fungi and *Thalassiosira pseudonana* and *Dictyostelium discoideum*, respectively. Three intron positions are shared between animals, plants, fungi and *Dictyostelium discoideum* and one including *Thalassiosira pseudonana* additionally.

Table 5.7 shows the phase distribution of shared intron positions. The majority of the positions are of the same phase (74.5%) in contrast to 25.5% of shared introns of different phases. In accordance with a prevalence of introns of phase 0, as shown in Figure 5.2, 43% of the shared intron positions are of phase 0.

Table 5.7: Phase distribution of shared intron positions in proteins of the oxidative phosphorylation pathway. The number of phases represent the number of shared intron positions.

same phase		different phase	
0,0	42	0,1	11
1,1	12	0,2	6
2,2	7	1,2	4
0,0,0	3		
1,1,1	7	0,1,2	1
2,2,2	1		
		0,1,1,1	1
		0,0,0,1	2
0,0,0,0,0	1		
total	73		25

5.2.3 Parallel intron gain in the *nad7* gene

A very interesting case of a shared intron position was found in the gene *nad7*. The nuclear encoded *nad7* gene of *Chlamydomonas reinhardtii* posesses 11 introns. One of these introns is located at the identical position within the nuclear *nad7* gene of animals. The observation, that this gene was transferred independently in the two divergent groups animals and plants (Figure 5.1), led to a more comprehensive analysis of the evolutionary history of this gene. In animal and fungal lineages studied to date, *nad7* is nuclear encoded, indicating that it was

transferred to the nucleus in the opisthokont common ancestor (Gray et al., 1999). Among higher plants, algae and protists, the gene is often still encoded in the mitochondrial genome, like in those of the green algae *Pseudendoclonium akinetum* (Pombert et al., 2004) and *Ostreococcus tauri* (Robbens et al., 2007). In contrast, *nad7* is absent from the mitochondrial genomes of the green algae *Chlamydomonas reinhardtii* and *Volvox carteri*, where it is nuclear encoded instead. The region of the shared intron position in the multiple sequence alignment is shown in Figure 5.5. The shared intron position is found in a very conserved region of the alignment, to which seven mitochondrial encoded *nad7* genes were added.

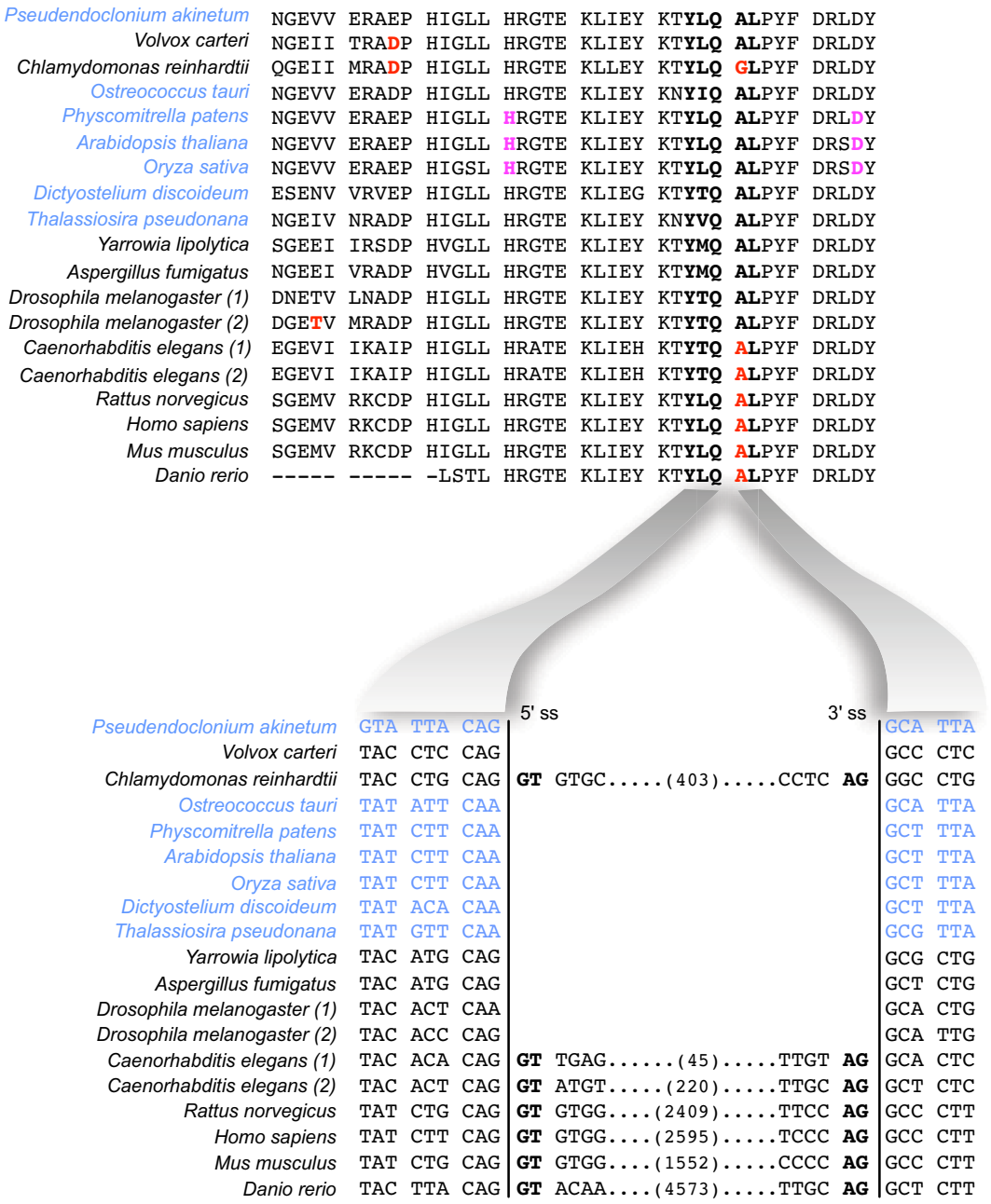


Figure 5.5: Shared intron position in the protein alignment of the gene *nad7*. A part of the multiple sequence alignment is shown, in which the mitochondrial encoded sequences are marked in blue, the nuclear enceoded sequences are marked in black. Spliceosomal introns are marked in red, the group II introns are marked in pink. The surrounding amino acids of the shared intron position between *Chlamydomonas reinhardtii* and the animals are shown at the nucleotide level, including the splicing sites and the length of the introns in parenthesis.

Altogether, there are four different group II intron positions present in the mitochondrial genes (Terasawa et al., 2007). Two of them are shared between *Arabidopsis thaliana* and *Oryza sativa* only. The other two introns are also present in *Physcomitrella patens* and surround the shared spliceosomal intron position, as shown in Figure 5.5. The closest group II intron resides 15 codons upstream and eight codons downstream regarding to the shared position in question. None of the mitochondrial introns are at the position of any spliceosomal intron. Figure 5.5 additionally shows the nucleotide region of the shared intron position in detail, including the 5´ and the 3´ splicing sites. The lenghts of the introns show an immense variation from 45 nucleotides in *Caenorhabditis elegans* up to 4573 nucleotides in *Danio rerio*. Three codons upstream and two codons downstream of the intron splicing sites are presented, revealing that all introns are of phase 0. If an intron is present, the last codon before the position is a CAG, as well as in all nuclear encoded sequences, with one exception in one of the two *Drosophila melanogaster* sequences. Also with one exception in the green alga *Pseudendoclonium akinetum*, in all mitochondrial sequences, the codon CAA is found at the position in question. The nucleotide sequences surrounding the independently inserted introns correspond to a classical protosplice site (Dibb and Newman, 1989), which is constituted of the pattern CAG/GC.

The endosymbiotic gene transfer to the nucleus in the two green algae *Chlamydomonas reinhardtii* and *Volvox carteri* happened independently of the opisthokont transfer, as evidenced by the common ancestry of the nuclear and mitochondrial copies within the chlorophyte lineage. The comprehensive phylogenetic tree, including the nuclear and mitochondrial encoded genes of *nad7* supports this assumption (Figure 5.6).

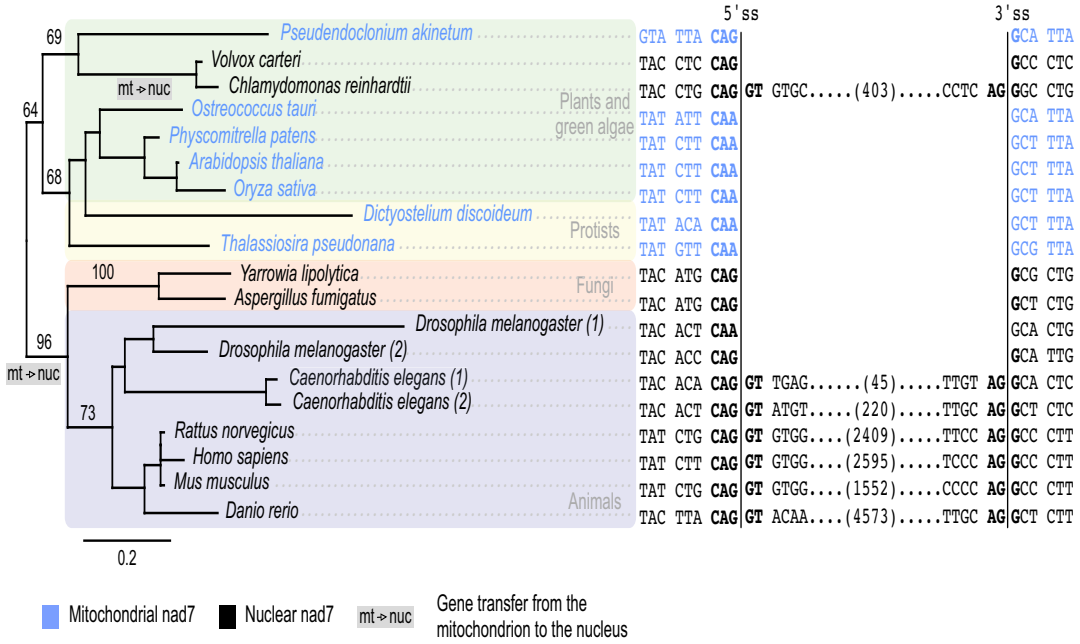


Figure 5.6: Phylogeny and identical intron position of the *nad7* gene. Species in which *nad7* is mitochondrial encoded are marked in blue, those in which it is nuclear encoded are marked in black. The bootstrap values and the endosymbiotic gene transfer of *nad7* are labelled at the branches of the tree. The region of the shared intron position is shown at the nucleotide level, including the splicing sites and the length of the introns in parenthesis.

The part of the multiple protein alignment shows a conserved region around the intron position. The different codons CAG and CAA upstream of the intron position encode the same amino acid glutamine (Q). The codon usage of glutamine is shown in Table 5.8 for the nuclear and the mitochondrial genome (Section 4.4.3). The percentages of used codons in the different species reveal a preference of the codon CAA with an average percentage of 74.6% in the mitochondrial genomes of *Dictyostelium discoideum, Thalassiosira pseudonana, Arabidopsis thaliana, Oryza sativa, Pseudendoclonium akinetum, Ostreococcus tauri* and *Physcomitrella patens*. In contrast, there is a higher average percentage of 69.6% of the codon use of CAG in the nuclear genomes of *Chlamydomonas reinhardtii, Volvox carteri, Caenorhabditis elegans, Drosophila melanogaster, Danio rerio, Homo sapiens, Rattus norvegicus, Mus musculus, Aspergillus fumigatus* and *Yarrowia lipolytica*.

Table 5.8: Preferred codon usage of the amino acid Glutamine in the mitochondrial genome (a) and the eukaryotic nucleus (b). Percentages are taken from the codon usage database[1] (Nakamura et al., 2000).

(a) Percentage of codon usage in the mitochondrial genome

Organism	Glutamine, CAA [%]	Glutamine, CAG [%]
Dictyostelium discoideum	89.3	10.7
Thalassiosira pseudonana	82.6	17.4
Arabidopsis thaliana	63.1	36.9
Oryza sativa	70.4	29.6
Pseudendoclonium akinetum	72.4	27.6
Ostreococcus tauri	81.9	18.1
Physcomitrella patens	81.4	16.6
Average codon usage	**74.8**	**25.2**

(b) Percentage of codon usage in the nucleus of eukaryotes

Organism	Glutamine, CAA [%]	Glutamine, CAG [%]
Chlamydomonas reinhardtii	10.4	89.6
Volvox carteri	26.7	73.3
Caenorhabditis elegans	65.6	34.4
Drosophila melanogaster	30.2	69.8
Danio rerio	26.0	74.0
Homo sapiens	26.5	73.5
Rattus norvegicus	24.7	75.3
Mus musculus	26.0	74.0
Aspergillus fumigatus	35.8	64.2
Yarrowia lipolytica	19.0	81.0
Average codon usage	**30.4**	**69.6**

[1]http://www.kazusa.or.jp/codon/

5.3 Ribosomal mitochondrial proteins

The proto-mitochondrial genes of the ribosomal mitochondrial proteins are listed in Table 5.9. According to Smits et al. (2007), the genes are chosen if they are present in *Homo sapiens* and if they have a homolog in *Rickettsia prowazekii*. Fol-

lowing the same procedure as used for the genes of the oxidative phosphorylation pathway, the mitochondrial gene names were identified by homology search in the mitochondrial genome of *Reclinomonas americana*. If the gene name could not be identified by homology search, the human SWISS-PROT gene name was listed in the table.

Table 5.9: Proto-mitochondrial ribosomal genes in *Homo sapiens* are listed with their SWISS-PROT identifier and the assigned mitochondrial gene names. *rplx, rpsx* are the mitochondrial gene names according to *Reclinomonas americana* (Section 4.1.1) while *MRPLx and MRPSx* are the SWISS-PROT gene names.

Large ribosomal subunit		Large subunit	
SWISS-PROT ID	mitochondrial gene name	SWISS-PROT ID	mitochondrial gene name
Q9BYD6	*rpl1*	Q9BYC8	*rpl32*
Q5T653	*rpl2*	O75394	*MRPL33*
P09001	*MRPL3*	Q9BQ48	*rpl34*
Q9BYD3	*MRPL4*	Q9NZE8	*MRPL35*
Q9BYD2	*MRPL9*	Q9P0J6	*MRPL36*
Q7Z7H8	*rpl10*	Q9BRJ2	*MRPL45*
Q9Y3B7	*rpl11*	Q9HD33	*MRPL47*
P52815	*MRPL12*	Small ribosomal subunit	
Q9BYD1	*MRPL13*	SWISS-PROT ID	mitochondrial gene name
Q6P1L8	*rpl14*	Q9Y399	*rps2*
Q9P015	*MRPL15*	P82675	*MRPS5*
Q9NX20	*rpl16*	P82932	*MRPS6*
Q9NRX2	*MRPL17*	Q9Y2R9	*rps7*
Q9H0U6	*rpl18*	P82933	*MRPS9*
P49406	*rpl19*	P82664	*rps10*
Q9BYC9	*rpl20*	P82912	*rps11*
Q7Z2W9	*MRPL21*	O15235	*rps12*
Q9NWU5	*MRPL22*	O60783	*rps14*
Q16540	*MRPL23*	P82914	*MRPS15*
Q96A35	*MRPL24*	Q9Y3D3	*MRPS16*
Q9P0M9	*rpl27*	Q9Y2R5	*MRPS17*
Q13084	*MRPL28*	P82921	*MRPS21*
Q8TCC3	*MRPL30*	Q96EL2	*MRPS24*

The different genomic locations of the mitochondrial ribosomal genes are shown in Table 5.10. Most of the mitochondrial ribosomal genes were transferred to the nucleus during evolution. Mitochondrial encoded genes are only found in the four species *Arabidopsis thaliana*, *Oryza sativa*, *Dictyostelium discoideum*, and *Thalassiosira pseudonana*. Some genes could not be identified by homology

search, others are lost completly from both, the mitochondrial and the nuclear genome. A loss of the genes *rps2*, *rps10*, and *rps11* in *Arabidopsis thaliana* is reported in Adams et al. (2002), indicated with "L" in Table 5.10.

Table 5.10: Genomic location of mitochondrial ribosomal genes.

Human Swiss-Prot ID	mitochondrial gene name	*Homo sapiens*	*Drosophila melanogaster*	*Danio rerio*	*Caenorhabditis elegans*	*Mus musculus*	*Rattus norvegicus*	*Aspergillus fumigatus*	*Yarrowia lipolytica*	*Candida glabrata*	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Chlamydomonas reinhardtii*	*Arabidopsis thaliana*	*Oryza sativa*	*Dictyostelium discoideum*	*Leishmania major*	*Plasmodium falciparum*	*Thalassiosira pseudonana*
Q9Y399	*rnc2*	N	N	N	N	N	N	N	N	N	N	N	-	**L**	**M**	**M**	-	-	**M**
P82675	MRPS5	N	N	N	N	N	N	N	N	N	N	N	-	N	N	-	-	-	N
P82932	MRPS6	N	N	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
Q9Y2R9	*rps7*	N	N	N	N	N	N	-	-	-	-	-	-	**M & N**	**M**	**M**	-	-	**M**
P82933	MRPS9	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
P82664	*rps10*	N	N	N	N	N	N	-	-	-	-	-	-	**L**	-	-	-	-	**M**
P82912	*rps11*	N	N	N	N	N	N	-	-	-	-	-	-	**L**	-	**M**	-	-	**M**
O15235	*rps12*	N	N	-	N	N	N	N	N	N	N	N	N	**M & N**	**M**	**M**	-	N	**M**
O60783	*rps14*	N	N	N	N	N	N	N	-	-	-	N	N	N	-	**M**	-	N	**M**
P82914	MRPS15	N	N	N	-	N	N	-	-	N	N	N	-	-	-	-	-	-	-
Q9Y3D3	MRPS16	N	N	N	N	N	N	N	N	N	N	-	-	N	N	-	-	-	N
Q9Y2R5	MRPS17	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
P82921	MRPS21	N	N	-	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q96EL2	MRPS24	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q9BYD6	*rpl1*	N	N	N	N	N	N	-	-	-	-	-	-	-	N	-	-	-	-
Q5T653	*rpl2*	N	N	N	N	N	N	N	N	N	N	N	N	**M**	**M**	**M**	-	N	**M**
P09001	MRPL3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Q9BYD3	MRPL4	N	N	N	N	N	N	N	-	-	N	N	N	N	N	N	-	-	N
Q9BYD2	MRPL9	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q7Z7H8	*rpl10*	N	N	N	-	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q9Y3B7	*rpl11*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	**M**	N	N	N
P52815	MRPL12	N	N	N	N	N	N	N	N	N	N	N	N	-	-	N	-	-	-
Q9BYD1	MRPL13	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Q6P1L8	*rpl14*	N	N	N	N	N	N	-	-	-	-	-	-	-	-	**M**	-	-	**M**
Q9P015	MRPL15	N	N	N	N	N	N	N	-	N	N	N	-	N	N	N	-	N	N
Q9NX20	*rpl16*	N	N	N	N	N	N	-	-	-	-	-	N	**M**	-	**M**	-	-	**M**
Q9NRX2	MRPL17	N	N	N	N	N	N	N	-	N	N	N	N	N	N	N	-	N	N
Q9H0U6	*rpl18*	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
P49406	*rpl19*	N	N	N	N	N	N	-	-	-	-	-	-	N	N	-	-	-	-
Q9BYC9	*rpl20*	N	N	-	-	N	N	-	-	-	-	-	N	N	N	N	-	-	N
Q7Z2W9	MRPL21	N	N	-	N	N	N	-	-	-	-	-	-	-	-	N	-	-	-
Q9NWU5	MRPL22	N	N	N	N	N	N	-	-	-	-	-	-	N	N	-	-	-	-
Q16540	MRPL23	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q96A35	MRPL24	N	N	N	N	N	N	-	-	-	-	-	N	N	N	N	N	N	N
Q9P0M9	*rpl27*	N	N	N	-	N	N	-	-	N	N	-	-	N	N	-	-	-	-
Q13084	MRPL28	N	N	N	N	N	N	-	-	-	-	N	-	N	-	N	-	-	-
Q8TCC3	MRPL30	N	N	N	-	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q9BYC8	*rpl32*	N	N	N	N	N	N	-	-	N	-	-	-	-	-	-	-	-	-
O75394	MRPL33	N	N	N	-	N	N	-	-	-	-	-	-	-	-	-	-	-	N
Q9BQ48	*rpl34*	N	-	N	-	N	N	-	-	N	N	N	-	-	-	-	-	-	-
Q9NZE8	MRPL35	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q9P0J6	MRPL36	N	N	-	-	N	N	-	-	-	N	N	-	-	-	-	-	-	-
Q9BRJ2	MRPL45	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-
Q9HD33	MRPL47	N	N	N	N	N	N	-	-	-	-	N	-	N	N	N	N	N	N

N – the gene is located in the nuclear genome of the species
M – the gene is located in the mitochondrial genome
L – the gene is lost in the species
'-' – the gene could not be identified with the homology search

The two genes *rps7* and *rps12* appeared to exist in both, the mitochondrial and the nuclear genome of *Arabidopsis thaliana*. These cases were verified and detailed information about the chromosomal locations with the corresponding accession number in NCBI is shown in Table 5.11. Both genes, located on chromosome 2 in *Arabidopsis thaliana* are annotated according to their expected function and show a 100% sequence identity to their mitochondrial encoded counterparts.

Table 5.11: Mitochondrial ribosomal genes encoded in the mitochondrial and the nuclear genome. The accession numbers are given for each gene and the definitions annotated in NCBI. The chromosomal location of the nuclear encoded gene and the pairwise identities between the mitochondrial and the nuclear genes computed by BLAST.

genomic location	Accession number (gene description)	pairwise identity
rps7 - Arabidopsis thaliana		
nucleus (chromosome 2)	NP_178787 (ribosomal protein S7 family protein)	100%
mitochondrion	NP_085579 (ribosomal protein S7)	
rps12 - Arabidopsis thaliana		
nucleus (chromosome 2)	NP_178773 (ribosomal protein S12 mitochondrial family protein)	100%
mitochondrion	NP_085552 (ribosomal protein S12)	

The procedure of identifying the time of the different transfer events is the same as shown for the genes of the oxidative phosphorylation pathway in Section 5.2. Endosymbiotic gene transfer events of mitochondrial ribosomal genes are labelled at the branches of the tree in Figure 5.7.

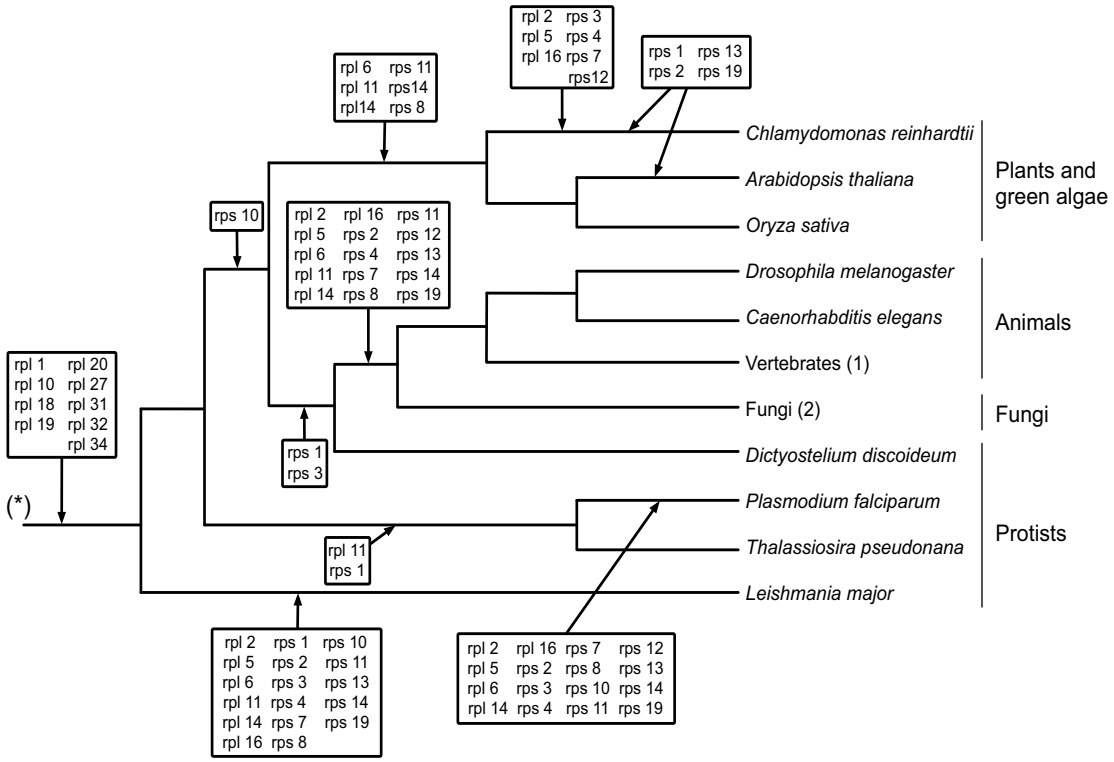
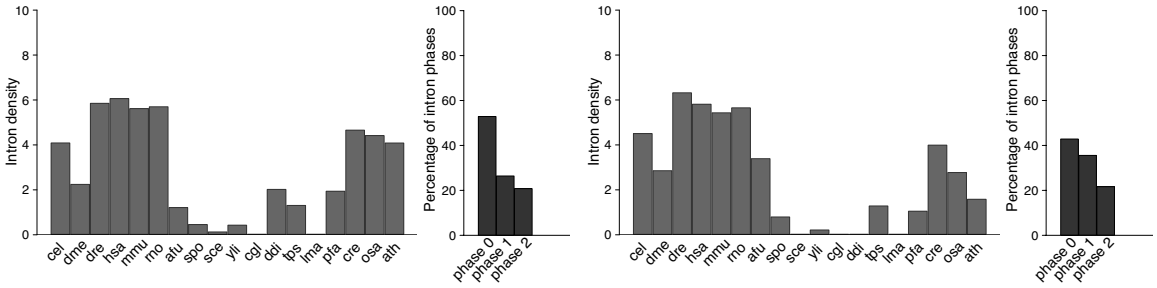


Figure 5.7: Timing of endosymbiotic gene transfer of proto-mitochondrial genes of ribosomal mitochondrial proteins. Gene transfer events are labelled at the branches. The tree represents the taxonomic relationship between the species. The time of transfer of a gene is relative to the speciation events depending on the presence/absence in the mitochondrial genome (Section 4.3). (1) *Danio rerio, Homo sapiens, Mus musculus, Rattus norvegicus* (2) *Aspergillus fumigatus, Schizosaccharomyces pombe, Yarrowia lipolytica, Saccharomyces cerevisiae, Candida glabrata*. (*) - bacteria and archaea.

5.3.1 Intron densities and phase distributions

The intron densities and the percentages of different intron phases are represented in Figure 5.8. The highest intron density with 6.07 and 5.92 introns per kilo basepairs coding sequence, is found in the vertebrates, in the species *Danio rerio* and *Homo sapiens*. The species *Rattus norvegicus*, *Arabidopsis thaliana*, and *Mus musculus* have almost the same intron densities of 5.66, 5.63, and 5.5 introns per kilo basepairs of coding sequence, respectively. The two species *Candida glabrata* and *Leishmania major* do not contain introns in genes of this dataset, as well as in their genes of the oxidative phosphorylation pathway (Figure 5.2).

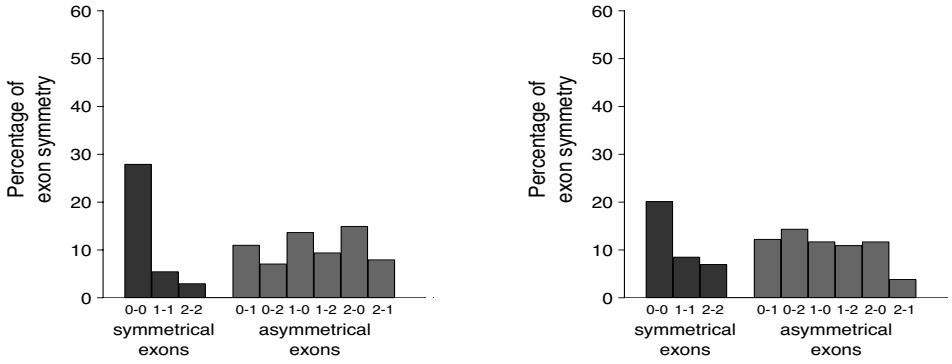


(a) Large mitochondrial ribosomal subunit (b) Small mitochondrial ribosomal subunit

Figure 5.8: Intron densities and phase distributions in genes for mitochondrial ribosomal proteins. Intron densities for each species in the genes of the two ribosomal subunits are shown with the percentages of intron phases. Intron density is given as the number of introns per kilo basepairs of coding sequence. cel=*Caenorhabditis elegans*, dme=*Drosophila melanogaster*, dre=*Danio rerio*, hsa=*Homo sapiens*, mmu=*Mus musculus*, rno=*Rattus norvegicus*, afu=*Aspergillus fumigatus*, spo=*Schizosaccharomyces pombe*, sce=*Saccharomyces cerevisiae*, yli=*Yarrowia lipolytica*, cgl=*Candida glabrata*, ddi=*Dictyostelium discoideum*, tps=*Thalassiosira pseudonana*, lma=*Leishmania major*, pfa=*Plasmodium falciparum*, cre=*Chlamydomonas reinhardtii*, osa=*Oryza sativa*, ath=*Arabidopsis thaliana*.

The percentages of introns of phase 0 (47.85%), phase 1 (30.96%), and phase 2 (21.195%), respectively result in a ratio of 5:3:2 as observed in the genes of the oxidative phosphorylation pathway (Section 5.2).

The number of symmetrical and asymmetrical exons is shown in Figure 5.9. Most of the exons are symmetrical 0-0 exons. In both ribosomal subunits, 23.9% of the exons are 0-0 symmetrical exons. The lowest percentage of asymmetrical exons corresponds to 2-1 exons with 5.8%.



(a) Large mitochondrial ribosomal subunit

(b) Small mitochondrial ribosomal subunit

Figure 5.9: Exon symmetry distribution in genes for mitochondrial ribosomal proteins of the large (a) and small (b) subunit.

Figure 5.10 shows the intron density of genes that were transferred from the mitochondrion at different evolutionary stages in the species *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*. The genes *rpl32* and *rpl19* are the most anciently transferred genes, followed by the genes *rps10* and *rps3*. The three genes *rpl16*, *rps7*, and *rps11* are the most recently transferred genes in the four species. The lowest intron density can be observed in *Drosophila melanogaster*. *Homo sapiens* and *Danio rerio* show very similar intron densities in all genes. Escpecially the two genes *rps10* and *rps11* show differing intron densities in the species. There is no correlation between the time of the transfer event and the intron density of the genes.

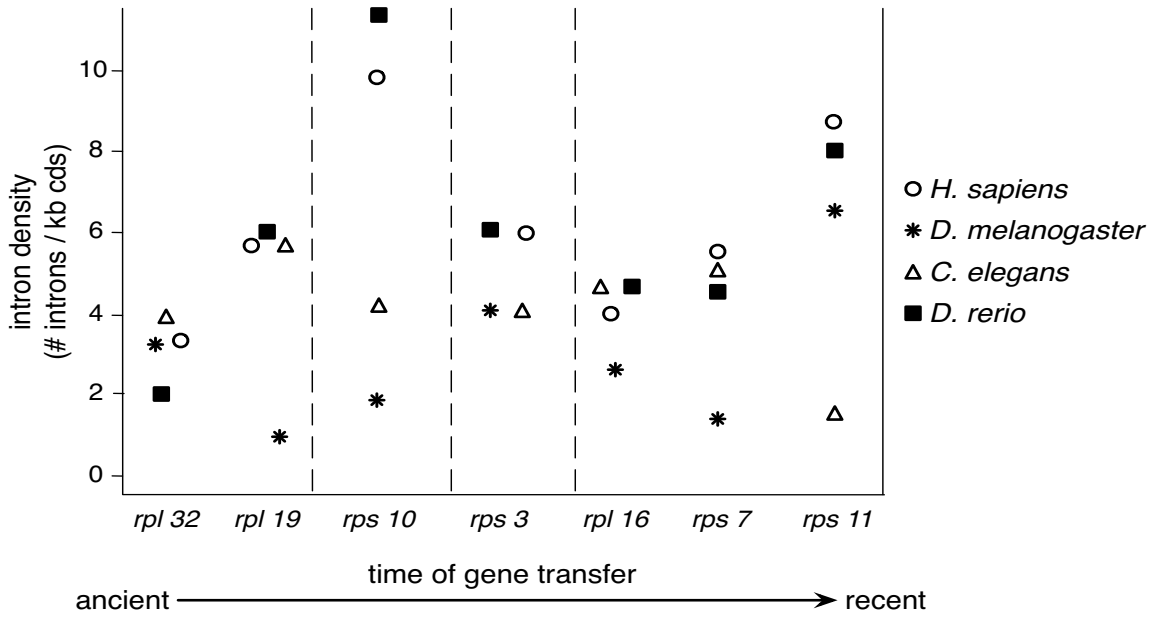


Figure 5.10: Intron density in genes transferred at different evolutionary stages in *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Drosophila melanogaster-Danio rerio*. Four different evolutionary times of endosymbiotic gene transfer are represented, with the two genes *rpl32* and *rpl19* as the most ancient gene transfers and the three genes *rpl16*, *rps7*, and *rps11* as the most recent gene transfer events in all four eukaryotes.

5.3.2 Shared intron positions

The number of shared intron positions between the different groups of species are listed in Table 5.12. The species specific intron positions are listed along the diagonal. Above the diagonal, the numbers represent the shared intron positions in the complete multiple alignments. Below the diagonal, the numbers represent the shared intron positions only in conserved regions of the alignments, determined with the program Blockmaker (Section 4.2.3).

Table 5.12: Shared intron positions in genes of mitochondrial ribosomal proteins. Shared intron positions between the different groups of species within the complete multiple protein alignments are shown above the diagonal, shared intron positions only within conserved regions of the alignment are shown below the diagonal. Species specific intron positions are shown in the diagonal.

	Animals	Plants	Fungi	D. dis	L. maj	P. fal	T. pse
Animals	**318**	12	4	3	-	-	2
Plants	6	**105**	1	-	-	-	-
Fungi	1	1	**17**	-	-	-	-
D. dis	1	-	-	**6**	-	-	-
L. maj	-	-	-	-	**0**	-	-
P. fal	-	-	-	-	-	**11**	-
T. pse	1	-	-	-	-	-	**3**

Animals - *Caenorhabditis elegans, Danio rerio, Drosophila melanogaster, Homo sapiens, Mus musculus, Rattus norvegicus*
Plants - *Arabidopsis thaliana, Chlamydomonas reinhardtii, Oryza sativa*
Fungi - *Aspergillus fumigatus, Candida glabrata, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Yarrowia lipolytica*
D. dis - *Dictyostelium discoideum*
L.maj - *Leishmania major*
P. fal - *Plasmodium falciparum*
T. pse - *Thalassiosira pseudonana*

Excluding shared intron positions in non-conserved alignment regions, reduces the numbers approximately by half. Most of the intron positions are shared between animals and plants, which is 54.55% in the complete alignment and 60% in the conserved regions. Five intron positions are shared between more than two species. Two of them are shared between the group of animals, plants and *Dictyostelium discoideum*. One of these intron positions is shared between animals, fungi, *Dictyostelium discoideum* and the other one is found in the group of animals, fungi and plants. The numbers of shared intron positions of the same or different phase were computed (Table 5.13).

Table 5.13: Phase distribution of shared intron positions of mitochondrial ribosomal proteins. The number of phases are the number of shared intron positions.

same phase		different phase	
0,0	12	0,1	3
1,1	4	0,2	1
2,2	2		
0,0,0	2		
1,1,1	1	0,0,1	1
2,2,2	1		
total	22		5

Most of the intron positions are of the same phase. The 12 shared intron positions of phase 0, which comprise 44.45% of all phases, do not represent the 12 shared intron positions between animals and plants (Table 5.12). There was no interspecies specific phase distribution of shared intron positions observed.

5.4 Intron positions in gene duplications

The homology search revealed cases of gene duplications. These genes appeared to have the same or different intron positions as illustrated for the gene *nad9* of the oxidative phosphorylation pathway as an example for a duplication without intron positions and a duplication with the same intron positions. The multiple protein alignment is shown in Figure 5.11. The species in the alignment were sorted and marked in colour according to their groups, the green alga *Chlamydomonas reinhardtii* in green, the animals in blue (*Caenorhabditis elegans, Drosphila melanogaster, Danio rerio, Homo sapiens, Rattus norvegicus* and *Mus musculus* and the fungi *Aspergillus fumigatus* and *Yarrowia lipolytica* in red. The intron positions are highlighted in red at the corresponding amino acids in the alignment. The fungal and the green algal sequences have a single intron position each, which are both not shared by any other species in the alignment. Four introns are shared between *Danio rerio*, *Homo sapiens*, *Rattus norvegicus*, and *Mus musculus*, three of them in the conserved regions of the alignment, highlighted in purple (Figure 5.11). One intron position is shared by all animal sequences except one of the sequences of *Mus musculus*. Two duplicated genes are found in *Mus musculus* and *Rattus novegicus*. The two genes of *Rattus norvegicus* have the same

intron positions whereas on of the gene duplicates of *Mus musculus* has no introns. The two duplicates are each grouped in one clade in the phylogenetic tree of Figure 5.12. In the maximum likelihood tree, the species are shown with their intron profiles which present the presence/absence of intron positions in the alignment.

The similarity between the intron profiles is graphically represented in the median network in Figure 5.13. In contrast to the phylogenetic tree (Figure 5.12) which is based on sequence similarity, the network represents the relationship between the species depending on their intron positions. The network reflects the number of introns in the species and the number of shared intron positions between the species. The sequences without or with only one intron appear as one group in the network, which are the sequences of *Yarrowia lipolytica*, *Mus musculus (2)*, *Aspergillus fumigatus* and *Chlamydomonas reinhardtii*, respectively. An internal split with a split weight of four (Figure 5.13) separates the species with a high number of introns from all other species. The external split of *Caenorhabditis elegans* also has a split weight of four, because there are four specific intron positions present in the alignment.

In the genes of the oxidative phosphorylation pathway, there were 14 cases identified in which an intronless duplication of a gene exists. In the mitochondrial ribosomal proteins this observation was made for 17 genes. In all of these cases, the duplicated genes are clearly identified on different chromosomal locations on the genome.

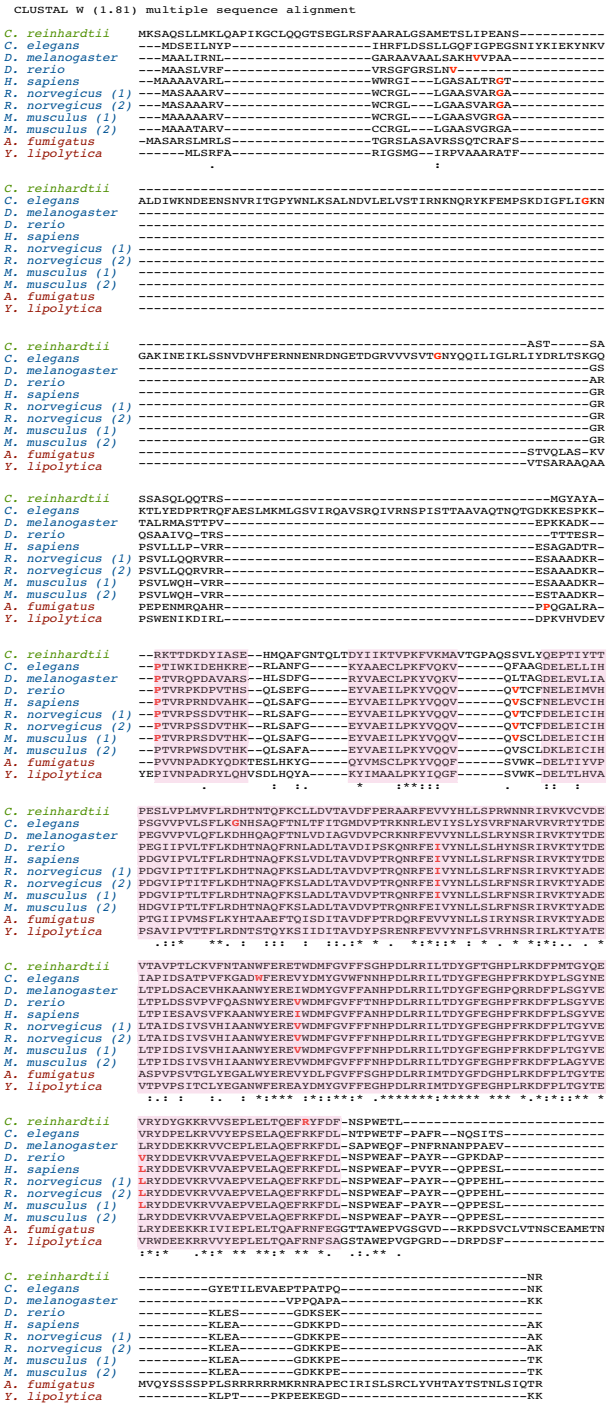


Figure 5.11: Multiple sequence alignment of the gene *nad9* of the oxidative phosphorylation pathway. The alignment was computed with MUSCLE and the coloured html alignment is shown in CLUSTAL-format. The intron positions are highlighted in red, the conserved regions determined with blockmaker are highlighted in purple. The species are coloured according to the groups green algae, animals and fungi in green, blue and red.

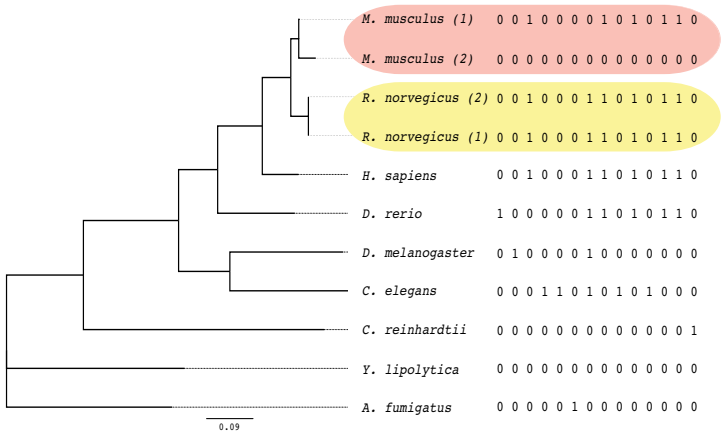


Figure 5.12: Phylogenetic maximum likelihood tree of the gene *nad9*. The tip labels are aligned at the end of the branches, showing the species and the intron profile for each sequence. The duplications of *Rattus norvegicus*, *Mus musculus* and *Caenorhabditis elegans* are labelled in yellow, red and green, respectively.

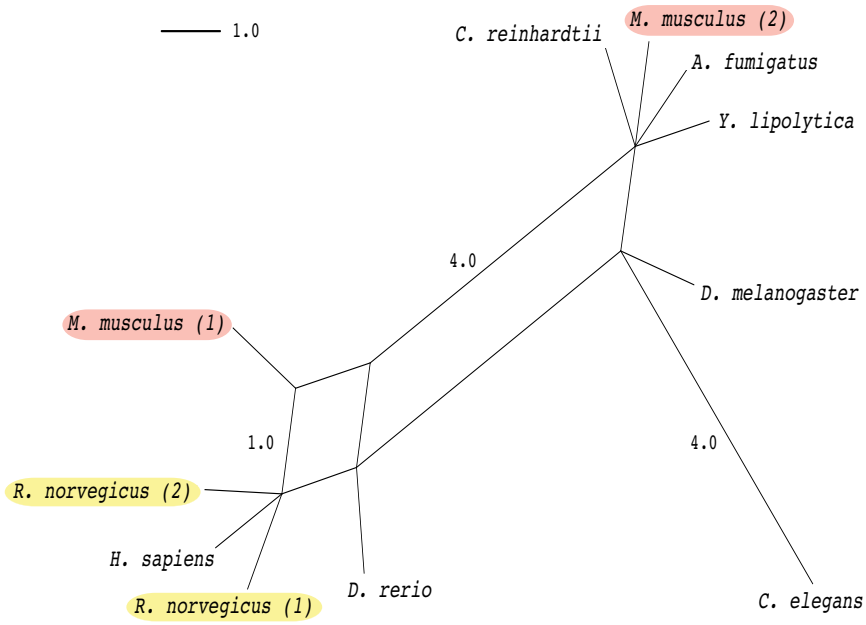


Figure 5.13: Median network of the intron profiles of the gene *nad9*. The duplications of *Rattus norvegicus* and *Mus musculus* are labelled in yellow and red, respectively. The split weights are labelled at the internal edges and if they differ from one.

6 Discussion

6.1 Database and annotation independent method to identify intron positions

The method presented in this work was developed to identify intron positions independently from gene annotations (Section 4.2.2). Genome and sequence annotations are not always reliable and complete. Using a secondary exon/intron database will limit the set of data that is included in primary databases. If a protein occurs to be intronless in a database, it is possible that this is due to missing annotation. Furthermore, the quality of annotations differ among genomes, depending on importance and time a species is studied. While investigations of the evolution of spliceosomal introns which include well annotated species and based on large scale analysis might not be significantly effected, a limited set of data under consideration may substantially reduce the reliability of exon/intron structure information and should therefore be treated carefully. Of course, exon/intron databases test the annotations as described in Section 4.2.1 for the EID (Exon-Intron Database) (Saxonov et al., 2000; Shepelev and Fedorov, 2006), but missing intron positions are not added.

The method developed here depends only on the quality of the sequences in the databases which are used. With a sufficient sequence quality a reliable identification of intron positions can be performed. Despite of being independent of annotation errors, the method developed in this work allows to identify intron positions in species or genes which are not included in exon/intron databases. Very recently, a similar method (Scipio) was published (Keller et al., 2008) in which also the program BLAT is used to align the protein sequences to their genomic counterparts. This publication supports the need for an annotation independent method to obtain information about the exon/intron structure of genes.

6.2 Endosymbiotic gene transfer, gene loss and sequence information

The timing of endosymbiotic gene transfer as described in Section 5.2 and Section 5.3 reveals a very dynamic evolutionary process with species specific gene transfer events at different evolutionary stages. The timing of the transfer events depends on the species phylogeny, the parsimonious method and on the available database information. This approach is similar to that, shown in Bonen and Calixte (2006), in which the timing of transfer events is inferred for the liverwort *Marchantia polymorpha*, and the plants *Arabidopsis thaliana* and *Oryza sativa* which leads to the same mitochondrial gene content of the two plants as identified in this work. But the separation of ancient and recent gene transfers becomes more differentiated with a growing number of species used here. The phylogenetic relationship of the 18 eukaryotes resolves in more detail independent gene transfers as in the case of the gene *nad7* which was transferred independently in the group of animals and the green alga *Chlamydomonas reinhardtii*. Observations of present ongoing gene transfer events are mainly reported for plant genomes (Adams et al., 2002; Bonen and Calixte, 2006), which is reflected in the results of this work. Most of the mitochondrial encoded genes are present in the two plants *Arabidopsis thaliana* and *Oryza sativa*, which provide the possibility of being transferred prospectively. The genes *atp1*, *atp9*, *rps7* and *rps12* are encoded in both, the mitochondrial and the nuclear genome of *Arabidopsis thaliana* which most probably represent recent endosymbiotic gene transfers. The presence of the gene *atp9* in *Aspergillus fumigatus* in both genomic locations cannot be clearly verified, because the presence of the gene in the mitochondrial genome is only proved for *Aspergillus niger*. But the presence of a nuclear encoded *atp9* gene in *Aspergillus niger* (Section 5.2) supports the existence of this gene in the mitochondrial and the nuclear genome in both lineages.

To have an equivalent basis of sequence similarity for the reconstruction of multiple sequence alignments, the human sequences were used as query sequences. In some cases, it was not possible to detect a homologous protein in a species where it should be present, given by the sources of Gabaldón et al. (2005) and Smits et al. (2007). Reasons for not detected genes by homology search (Table 5.4, Table 5.10) could be the restrictions of the BLAST search, missing sequence information in the database or an eventual gene loss. Allowing to

include missing sequences with a low sequence similarity would lead to problems in alignment reconstruction and consequently to problems in the identification of shared intron positions. So it was decided to keep the human proteins as query sequences and the e-value threshold of 10^{-6} for the filtering of the BLAST results.

Cases of gene loss cannot clearly be evidenced in this study. The experimentally verified cases of gene loss are reflected in the survey of endosymbiotic gene transfer (Table 5.4, Table 5.10). The main reason for mitochondrial gene loss, next to functional substitution, is the gene transfer to the nucleus (Adams et al., 2002; Adams and Palmer, 2003). Lost genes are included to represent a gene transfer as well as the other genes (Figure 5.1, Figure 5.7), because most probably they were transferred to the nuclear genome and subsequently lost from both, the mitochondrial and the nuclear genome.

6.3 Shared intron positions are not always ancient

The number of shared intron positions in homologous genes are mainly discussed under the terms of the introns-early and the introns-late hypothesis where shared positions are either conserved or they can be gained independently at the same site in the gene, respectively. Most shared intron positions are found between the most divergent groups animals and plants (Fedorov et al., 2002; Rogozin et al., 2003) giving rise to the assumption that these positions were achieved in the common ancestor of these species.

Taking into account the shared intron positions only in conserved regions of the alignments, lead to 62.5% of shared intron positions between animals and plants. This number is twice of the percentage, reported in other studies, where 25-30% of intron positions are shared between animals and plants (Sverdlov et al., 2005).

A clear case of a parallel intron gain was identified in the gene *nad7* of the oxidative phosphorylation pathway (Section 5.2.3). Although the mitochondrial encoded *nad7* genes of higher plants contain four group II introns (Terasawa et al., 2007), the molecular ancestors of spliceosomal introns (Martin and Koonin, 2006; Toro et al., 2007), none are present at the same position as the spliceosomal introns (Figure 5.5). Given the absence of spliceosomes in mitochondria, the present data indicate that the nuclear *nad7* intron shared by animals and *Chlamy-*

domonas reinhardtii has been acquired by independent insertions at indentical positions in those lineages. The nuclear codon usage might have facilitated intron insertion and efficient splicing at this site (Schwartz et al., 2008). Arguments in favor of intron antiquity at identical intron positions are traditionally founded in weighting the relative probabilities of massive intron loss versus a few parallel intron gains (Sverdlov et al., 2005; Roy and Gilbert, 2006), with clear evidence for the existence of the latter lacking so far. The independent intron insertions in *nad7* following its independent transfers from the mitochondrion to the nucleus show that independent intron insertions do occur. While there can be no doubt that the last common ancestor of present eukaryotes had spliceosomes and introns (Jeffares et al., 2006; Martin and Koonin, 2006; Toro et al., 2007), the *nad7* gene of *Chlamydomonas reinhardtii* offers a straightforward counterexample to the view that identical intron positions reflect ancient conservation of intron positions.

Different contributions of parallel gained introns in contrast to the conservation of shared intron positions are reported. In Qiu et al. (2004) most shared intron positions should be inserted at the same sites independently. A percentage of 5-10% of shared intron positions as a result of parallel intron gains is reported in Sverdlov et al. (2005). A similar number of 8% is reported as parallel intron gain using a probabilistic model (Carmel et al., 2007). 18% of shared intron positions are identified as parallel gains, computed with a maximum likelihood method (Nguyen et al., 2005). In this work, the parallel intron gain in the gene *nad7*, leads to 6.25% of parallel intron gain in the proteins under consideration. Clear examples of parallel intron gains as presented in this work are not easy to identify because the evolutionary history of most genes is not as clear as for the gene *nad7*.

6.4 Dynamic intron evolution in proto-mitochondrial genes

The intron characteristics in this study reveal a highly dynamic evolution of spliceosomal introns in genes that originated from endosymbiotic gene transfer from mitochondria. This conclusion is based on the observation that the intron characteristics in these genes are not significantly different from characteristics

of introns in ancestral eukaryotic genes. The distribution of intron densitiy among species (Figure 5.2, Figure 5.8) is in accordance with the intron density reported in the literature (Figure 3.5). Based on the number of identified introns in this dataset, the average number of introns per gene is 2.74 which represents the mean number of introns in the species of Figure 3.5.

A bias of phase 0 introns is a frequent observartion and often linked with the preference of newly gained introns (Long and Deutsch, 1999; Lynch, 2002). A ratio of 5:3:2 of phase 0, phase 1 and phase 2 introns as found in the genes of the oxidative phosphorylation pathway, as well as in the genes of mitochondrial ribosomal proteins (Section 5.2.1, Section 5.3.1) is in accordance with results reported for genes that did not originate by endosymbiotic gene transfer (Qiu et al., 2004; Ruvinsky and Ward, 2006). The 0-0 exons account for the majority of symmetrical exons (Figure 5.3, Figure 5.9). This observation is also reported among eukaryotic genomes (Ruvinsky and Ward, 2006).

The timing of endosymbiotic gene transfer events among the 18 eukaryotes enabled to test if the time of the gene origin takes influence on the intron density. It is possible, that more recently transferred genes have a lower intron density than genes that were transferred earlier in the eukaryotic evolution because of the higher possibility of intron accumulation over time. However, the genes of the oxidative phosphorylation pathway as well as the ribosomal mitochondrial genes, do not display correlation between the time of the transfer and the intron density. In Basu et al. (2008) there was a slightly but significantly lower intron density observed in nuclear encoded genes that originated by endosymbiotic gene transfer from the chloroplast than in ancestral eukaryotic genes what could be the result of the more recent endosymbiotic event that gave rise to chloroplasts around 1.5 billion years ago, in contrast to the preceding origin of mitochondria, around 2 billion years ago (Martin et al., 2003). Very recent transfer events in this analysis are most probably the genes *atp1*, *atp9*, *rps7*, and *rps12* because they are present in the mitochondrial and the nuclear genomes of *Arabidopsis thaliana*. Only the nuclear encoded gene *atp1* in *Arabidopsis thaliana* contains one intron, whereas the other genes are intronless which is in accordance with the observation in Basu et al. (2008), because these recently transferred genes reflect the low intron density. But a general lower intron desity cannot be identified in this data.

The recent transfers as well as the cases of intronless gene duplications

within species, count for preferred molecular mechanisms which do not retain introns in the transferred or duplicated genes. The genes which have an intronless copy in the genome, most probably arose by retrotransposition, one of the two main mechanisms of gene duplication as described in Zhang (2003), in which the processed/mature mRNA is reverse transcribed into DNA and subsequently inserted into the genome. The absence of introns in a proto-mitochondrial gene in the nucleus might not be an adequate evidence for determinig the mechanism of the transfer. The mechanism does not have to involve processed mRNA, because self-splicing introns which have been present at the time of the transfer could also be spliced out of the transcript after the integration into the nucleus (Henze and Martin, 2001).

A possibility to describe the different influences on the evolution of spliceosomal introns we can observe in this study is shown in Figure 6.1. Similar to the diagram in Jeffares et al. (2006), Figure 6.1 shows that mostly species specific factors determine the dynamics of intron evolution in genes that originated by endosymbiotic gene transfer from the mitochondrion. This is indicated by the intron density which is specific according to the overall intron density in each species. The time of the origin of the genes in the nuclear genome set the starting point at which spliceosomal introns can be gained, but as there is no correlation found between the time of the transfer and the intron density, the structure of genes seems to play a more prominent role.

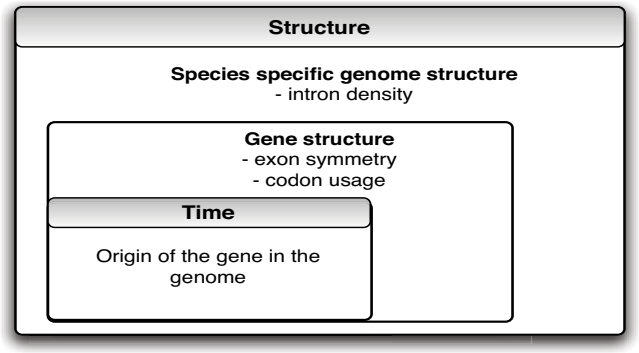


Figure 6.1: Influences on the dynamics of intron evolution in genes that originated by endosymbiotic gene transfer from the mitochondrion. The species specific genome structure is of major importance, which is reflected in the mean intron density of the species. The gene structure takes influence on intron gain represented in the symmetry of exons and the codon usage, which set restrictions to possible intron insertion sites. The time of the origin of the mitochondrial gene in the nuclear genome was identified to have fewest of all influence on the evolution of spliceosomal introns in these genes. (Based on Jeffares et al. (2006)).

6.5 Can we solve the question about the origin of spliceosomal introns?

The question about the origin of spliceosomal introns is not resolved yet. Results obtained from this study supports the introns-late hypothesis because all gained introns in the genes that originated by endosymbiotic gene transfer support a dynamic intron evolution with a high rate of intron gain, in contrast to a conservation of all introns that are present in eukaryotic genomes. The introns-late hypothesis sometimes mislead to the notion that introns originated only very late in evolution, but if we date the origin of spliceosomal introns to the origin of mitochondria, the timing "late" refers to the early eukaryotic evolution around 2 billion years ago (Martin et al., 2003).

The proposed evolutionary relationship between group II introns and spliceosomal introns gives an explanation for the origin of the spliceosome (Rodríguez-Trelles et al., 2006; Martin and Koonin, 2006). However, an evolutionary relationship between group II introns and spliceosomal introns could not be detected in this analysis. Some mitochondrial genomes do not contain group II introns at all, for example the mitochondrion of *Caenorhabditis elegans* (Coghlan and Wolfe,

2004), so that they cannot be responsible for recent intron gain we observe in genes that originated from endosymbiotic gene transfer. The observation that the group II introns of the mitochondrial encoded *nad7* gene are not at the same position as the spliceosomal introns in the nuclear encoded gene (Section 5.2.3) does not disprove their possible evolutionary relationship, especially since similarities between group II introns and the spliceosome are reported in recent studies (Valadkhan, 2007; Toro et al., 2007; Toor et al., 2008).

Inferred rates of intron gain today are too slow to explain the proliferation of introns with the current mechanisms (Roy and Gilbert, 2005, 2006), and consequentially, these rates are too slow to explain the intron density in genes that originated by endosymbiotic gene transfer, which gained introns in a relatively short period of time. The existing scenarios of intron evolution which are inferred under different assumptions and evolutionary models are too static to explain the dynamics of intron evlolution in all eukaryotic genes with different evolutionary histories. Also single case studies of parallel intron insertions do not lead to general trends or patterns in eukaryotic intron evolution. The results obtained in this work, support a continuously intron gain and loss during eukaryotic evolution including both, conservation of intron positions and parallel intron gains. According to Martin and Koonin (2006), the results would speak for a time in eukaryotic evolution in which the cell had adapt to the invading genes from the mitochondrion and the proliferation of introns in the genome, in which the development of a nucleus played an important role. Testing that hypothesis is, however, complicated by the overriding effects of lineage-specific gain and loss, the dynamics of which were uncovered in the work presented here.

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Eine, die du siehst,
Eine, die ich sehe,
Und eine, die wir beide nicht sehen.

(Chinesisches Sprichwort)