Fungal Genetics and Biology 62 (2014) 34–42

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Comparative analysis of mitochondrial genomes from closely related *Rhynchosporium* species reveals extensive intron invasion

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ARTICLE INFO

Article history:
Received 11 June 2013
Accepted 1 November 2013
Available online 13 November 2013

Keywords:
Genome evolution
Genome architecture
Intron encoded proteins
PAC species
Horizontal intron transfer

ABSTRACT

We sequenced and annotated the complete mitochondrial (mt) genomes of four closely related *Rhynchosporium* species that diverged ~14,000–35,000 years ago. During this time frame, three of the mt genomes expanded significantly due to an invasion of introns into three genes (*cox1*, *cox2*, and *nad5*). The enlarged mt genomes contained ~40% introns compared to 8.1% in uninvaded relatives. Many intron gains were accompanied by co-conversion of flanking exonic regions. The comparative analysis revealed a highly variable set of non-intronic, free-standing ORFs of unknown function (uORFs). This is consistent with a rapidly evolving accessory compartment in the mt genome of these closely related species. Only one free-standing uORF was shared among all mt genomes analyzed. This uORF had a mutation rate similar to the core mt protein-encoding genes, suggesting conservation of function among the species. The nucleotide composition of the core protein-encoding genes significantly differed from those of introns and uORFs. The mt mutation rate was 77 times higher than the nuclear mutation rate, indicating that the phylogeny inferred from mt genes may better resolve the phylogenetic relationships among closely related *Rhynchosporium* species than phylogenies inferred from nuclear genes.

1. Introduction

The principal function of mitochondria is to generate energy, mainly by oxidative phosphorylation (Saraste, 1999). In addition to their central role in ATP production, mitochondria play important roles in intermediary metabolism, ion homeostasis and apoptosis (Burger et al., 2003). The restricted number of genes encoded by mitochondrial (mt) genomes is a result of the extensive translocation of genes to the nucleus that followed endosymbiosis of the original bacterial ancestor (Adams et al., 2000; John and Whatley, 1975). Indeed most mt proteins are encoded by nuclear (nu) genes and subsequently imported into the mitochondrion (Herrmann, 2003). Mt genomes typically encode 14 proteins related to the respiratory chain complexes, two ribosomal subunits, a distinct set of tRNAs and a variable number of free-standing open reading frames of unknown function (uORFs) (Gray, 1999; Lang et al., 1999). uORFs are poorly conserved across species and their role is not clear. Most mt genomes are uniparentally inherited, lack methylation, show highly conserved core protein-encoding genes, and are present in many copies within each cell (Campbell et al., 1999; Chen and Hebert, 1999). Because of these properties, mt gene sequences have been widely used as markers for population and species characterization (Moore, 1995). Mt genomes have also been used to study the evolution of organelles (Gray, 1999), fungicide resistance (Torriani et al., 2009) and gene structure (Jurica and Stoddard, 1999; Sanchez-Puerta et al., 2008).

Up to now 119 fungal mt genomes have been deposited in the publicly available NCBI database. Mt genomes are normally reported as circular molecules characterized by high AT-content. However there is strong evidence that several circular mapping mt genomes consist of linear concatemeres (Burger et al., 2003; Jacobs et al., 1996; Maleszka and Clark-Walker, 1992). Fungal mt genomes range in size from 18.8 kb (*Hanseniaspora uvarum*) to 156.3 kb (*Phlebia radiata*) with an average of 48.5 kb (Organelle Genome Resource Database of NCBI). Differences in the length and organization of intergenic regions combined with the variable number and size of introns explain most of the variability in mt genome size (Burger et al., 2003). Plant and fungal mt genomes often contain large introns up to 5 kb in size, while those of Metazoa rarely have introns (Lang et al., 2007). Mt genomes of fungi differ significantly in intron content, e.g. in *Podospora anserina* about

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1087-1845
http://dx.doi.org/10.1016/j.fgb.2013.11.001

75% of the mt genome is composed of introns (Cummings et al., 1990), while the mt genomes of *Zymoseptoria tritici* (syn *Mycosphaerella graminicola*) and *Phialocephala fortinii* are intron-free (Duò et al., 2012; Torriani et al., 2008). A recent analysis of introns in the mt gene *cox1* from 640 angiosperms concluded that these introns were acquired via ~70 distinct horizontal transfers and that the first donor was most likely a fungus (Sanchez-Puerta et al., 2008). Because of the limited availability of complete mt genome sequences from closely related species, it has been difficult to estimate the timeframes associated with intron fixation or invasion of introns into mt genes or genomes. Comparative analysis of nu genomes from closely related species already provided new insights into the evolutionary mechanisms associated with nu intron gain and loss (Torriani et al., 2011; Li et al., 2009).

Rhynchosporium spp. belong to the Leotiales and are closely related to the genera Pyrenopeziza and Tapesia. They are related to several anamorphic fungi, including *Phialophora aggregata* and *Phialocephala subalpina* (Duò et al., 2012; Goodwin, 2002). The cultivated cereals barley, rye and triticale, as well as grasses belonging to the genera *Agropyron*, *Bromus*, *Dactylis*, *Hordeum* and *Lolium* are among the plant hosts of *Rhynchosporium* spp. (Caldwell, 1937; Shipton et al., 1974; Welty and Metzger, 1996; Zaffarano et al., 2011). Recently a multilocus phylogeny inferred by combining three nu loci (α-tubulin, β-tubulin and ITS) and using 316 *Rhynchosporium* isolates sampled from cultivated and uncultivated hosts resulted in four host-specialized monophyletic groups. *Rhynchosporium commune* was found on barley, other *Hordeum* spp. and *Bromus diandrus*. *Rhynchosporium secalis* was found on rye and triticale, *Rhynchosporium agropyri* was found on *Agropyron* spp. and *Rhynchosporium orthosporum* infected *Dactylis glomerata* (Zaffarano et al., 2011, 2009). No cross-pathogenicity was found using a pathogenicity assay that included 24 *Rhynchosporium* isolates from different phylogenetic lineages and using barley, rye and *Hordeum murinum* as hosts (Zaffarano et al., 2008).

In this study we assembled, annotated and analyzed the complete mt genomes of *R. commune*, *R. secalis*, *R. agropyri* and *R. orthosporum*. We applied the Phylogenetic Species Concept (PSC) to the mt genome sequences to test the *Rhynchosporium* spp. boundaries that were defined based on nu markers (Zaffarano et al., 2011). We also compared the mutation rates of mt and nu genes to test the hypothesis that fungal mt genomes evolve faster than nu genomes (Burger et al., 2003; Ghikas et al., 2010). Up until now, relatively few studies compared the mt genomes of closely related fungal species. Duò et al. (2012) analyzed eight members of the *P. fortinii* s.l. – *Acephala applanata* species complex (PAC), revealing a variable set of free-standing mt uORFs. Similar sets of uORFs were reported when comparing two *Glomus* spp. (Beaudet et al., 2013), three *Fusarium* spp. (Al-Reedy et al., 2012), six *Aspergillus* spp. and three *Penicillium* spp. (Joardar et al., 2012). In general, uORF homologs are rarely shared between different species, but a few uORFs shared among *Fusarium* spp. showed signatures of positive selection (Al-Reedy et al., 2012). Comparative analyses of closely related species showed that the major sources of mitochondrial genome size variation were differences in the number and size of introns in the core mt protein-encoding genes and differences in uORF complements (Al-Reedy et al., 2012; Beaudet et al., 2013; Joardar et al., 2012). Although the functions and evolutionary origins of mt uORFs and introns are unclear, their presence illustrates the plasticity of fungal mt genomes.

2. Materials and methods

2.1. Fungal strains and DNA extraction

Rhynchosporium isolates 02CH4-6a.1 (*R. secalis*), 04CH-RAC-A.6.1 (*R. agropyri*) and 04CH-BAR-A.1.1.3 (*R. orthosporum*) were collected in Switzerland (Zaffarano et al., 2008), whereas *R. commune* isolate UK7 originated in the United Kingdom (Lehnackers and Knogge, 1990). Fungal mycelia were grown in 145-mm Petri dishes in Fries medium No. 3, supplemented with sucrose and yeast extract, as described previously (Rohe et al., 1996). For extraction of total DNA, 10-d old mycelia were filter-dried and ground with mortar and pestle in liquid nitrogen. The powder was resuspended in 1 vol. of 100 mM Tris/HCl (1% sarcosyl, 100 mM NaCl, 10 mM EDTA, 1% (w/v) polyvinylpyrrolidone), pH 8.0. RNA was removed by incubating the suspension with RNase A (300 μg/ml) for 15 min at 37 °C. DNA was extracted by adding 0.2 vol. of phenol/$CHCl_3$/isoamyl alcohol (25:24:1) to the suspension, shaking for 15 min at room temperature and centrifugation (15 min, 4 °C, 4000 rpm in an Eppendorf centrifuge 5810 R with swing-out rotor A-4-62). For DNA precipitation 0.55-ml aliquots of the upper phase were transferred to 2-ml Eppendorf tubes containing 1 ml of isopropanol. After mixing by inversion, the DNA was precipitated by centrifugation (10 min, 4 °C, 12,000 rpm in an Eppendorf centrifuge 5804 R with fixed-angle rotor F45-30-11). DNA pellets were washed with 70% (v/v) EtOH, air-dried and resuspended in water. DNA concentrations were determined using the NanoDrop 8000 spectrophotometer (ThermoScientific, Dreieich, Germany).

2.2. DNA sequencing and assembly

Nu and mt genomes of the fungal isolates were sequenced using a whole-genome shotgun sequencing approach. To overcome the difficulties associated with extremely high sequence depths (500-fold to >8000-fold) of the mt sequences, all *de novo* assemblies of mt genomes were performed using reduced read counts. For *R. commune* and *R. secalis* whole-genome 454 libraries were generated according to the manufacturer's protocol and sequenced using a Roche/454 GS FLX sequencer. This provided for *R. commune* ~2.37 million (891 Mb) and for *R. secalis* ~1.86 million (745 Mb) 454 sequences corresponding to a ~16-fold and ~15-fold haploid genome coverage, respectively. Approximately 5.0–5.6% of the reads represented mtDNA. For both isolates mt genomes were *de novo* assembled species-specifically from 100,000 randomly chosen reads using gsAssembler (Newbler v.2.3, 454 Life Sciences Corporation) with its standard parameters. Putative mt contigs/sequences were identified by homology searches against known mt fungal sequences using BlastN. To close the circular genomes and fill remaining gaps, all 454 reads were mapped back to the assembled sequences (Newbler). Additionally, pairs of primers were designed to amplify the missing regions. The amplicons were sequenced directly using Sanger technology to obtain the complete mt genomes of both isolates.

For *R. orthosporum* and *R. agropyri* paired-end (fragment size ~380 bp) libraries were generated from fragmented genomic DNA. Libraries were sequenced using Illumina GAII technology. For each of the isolates >32 million read pairs (6.5–7.6 Gb) were generated corresponding to >130-fold haploid genome coverage. In both cases, approximately 5.3–7.2% of the reads were derived from mtDNA. Mt genomes were *de novo* assembled using Velvet (v0.7.55). Assemblies were carried out with different numbers of randomly chosen reads (70,000; 140,000; 200,000; 400,000; 800,000; 1,600,000) and different *K*-mer lengths ($K = 45, 51, 61, 65, 71$). Best results were obtained using 400,000 reads and $K = 65$ (*R. orthosporum*) and 800,000 reads and $K = 51$ (*R. agropyri*), respectively. Assemblies were validated and putative mt contigs/sequences were identified using BlastN alignments against mt reference sequences. All remaining gaps were closed by remapping Illumina paired-end reads to their respective mt genome (Newbler). Additionally, for *R. orthosporum* and *R. agropyri* direct Sanger sequencing was applied as described above to obtain the entire mt genomes.

2.3. Sequence annotation

The mt genomes of all four *Rhynchosporium* spp. were screened for similarity with the existing fungal mt genomes deposited in NCBI. Database searches were performed using the NCBI BLAST algorithm (Altschul et al., 1990). In addition to the standard genes encoding proteins involved in oxidative phosphorylation, additional ORFs larger than 300 bp were annotated. Putative functions of uORF proteins were tested using BLASTP (Altschul et al., 1990) and INTERPROSCAN (Zdobnov and Apweiler, 2001). The mt gene sequences were aligned using Geneious v5.5 (Drummond et al., 2011) and manually curated to identify all polymorphisms and to create mt genome maps. *Cox1* was used as an anchor to linearize the four *Rhynchosporium* mt genomes and align them with the mt genome of the outgroup *P. subalpina* (JN031566; (Duò et al., 2012). Intron boundaries were confirmed by alignment of the predicted genes to *R. commune* EST sequences when available or through sequence comparisons with other fungal mt genomes. Introns were categorized by blasting the sequences to RNAweasel (Lang et al., 2007). The genes *rnl* and *rns* were predicted through sequence comparisons using BLASTN. The tRNAs were defined with tRNAscan-SE v.1.21 using the default settings for mt genomes (Lowe and Eddy, 1997). Nucleotide compositions of the complete mt genomes, exons, introns and free-standing uORFs were assessed using Geneious v.5.5 (Drummond et al., 2011). The presence of repetitive elements was analyzed using the program perfect microsatellite repeat finder (http://sgdp.iop.kcl.ac.uk/nikammar/repeatfinder.html), using the default settings.

2.4. Mutation rate estimates and interspecific comparisons

Zaffarano et al. (2008) used the program BEAST 1.4 (Drummond et al., 2005) to estimate Times to the Most Recent Common Ancestor (TMRCA) of *Rhynchosporium* species, using variation at nu genes based on previously published fungal mutation rates (Kasuga et al., 2002). To allow a direct comparison of rates of evolution of nu and mt genes in the *Rhynchosporium* spp. complex, we used the same version of BEAST to obtain Bayesian estimates of mt genome mutation rates using these inferred TMRCA as calibration points in a phylogenetic analysis. The calibration was based on the mean estimates, including upper and lower 95% highest posterior density (HPD) values provided in Table 2 of Zaffarano et al. (2008). All estimates assumed a lognormal relaxed clock model because this model scored significantly better than the other implemented models according to Bayes factor comparisons. The concatenated nucleotide sequences of 13 core mt genes (*atp6*, *atp8*, *cox1* to *cox3*, *cytb*, *nad1* to *nad6* and *nad4L*) were included in the analysis, using partition information to allow separate parameter estimates for each gene. The final Bayesian Markov Chain Monte Carlo (MCMC) analyses were run for 10^8 generations sampling every 1000th iteration. The performance of the MCMC process was checked for stationarity and sufficiently large effective sample sizes using TRACER (Drummond and Rambaut, 2007).

The nu phylogeny of the *Rhynchosporium* spp. was reconstructed using the same approach based on the concatenated alpha-tubulin, beta-tubulin and ITS sequences described previously (Zaffarano et al., 2008). Differences in phylogenetic relationships among *Rhynchosporium* spp. were tested by forcing species-pairs to form sister species, e.g. *R. secalis* and *R. agropyri*, *R. secalis* and *R. commune*, and *R. agropyri* and *R. commune*. The pairwise combinations were compared using the Bayes scores from the resulting trees.

The core protein-encoding genes from the four *Rhynchosporium* spp. mt genomes were compared to identify synonymous, non-synonymous and insertion/deletion (indel) mutations, as well as to measure nucleotide diversity (Pi) and sequence conservation (SC). These results were generated using the program DnaSP 5.10.01 (Librado and Rozas, 2009). The total sites refer to the number of nucleotides that could be compared in all species (indel or missing data were not considered). The numbers of synonymous and non-synonymous mutations were the totals over all possible pairwise comparisons. Pi is the average number of nucleotide differences per site between two sequences and SC represents the proportion of conserved sites in the alignment.

We tested competing evolutionary scenarios leading to the observed intron presence/absence polymorphisms. Because of the complexity and poor annotation quality of *cox1*, we restricted these analyses to *cox2*, which had unambiguous intron sequence alignments. (i) We compared the hypothesis that the hypothetical ancestor of *cox2* was intron-free with the hypothesis that the ancestral *cox2* possessed all three introns that we observed in *R. commune*, *R. secalis* and *R. agropyri*. To do this, we retrieved the most similar *cox2* sequences from GenBank using a BLAST search. We added these samples to our *Rhynchosporium* data set and used the program BEAST v1.7.1 (Drummond and Rambaut, 2007) to reconstruct an unconstrained genealogical relationship including all samples. Assuming that the common ancestor (i.e. the root of the tree) possessed or lacked the introns, we could then count backwards to infer the number of intron losses or gains. This simplistic but parsimonious approach assumes that there were intron losses if the ancestor possessed the intron, or intron gains if the ancestor lacked the introns. (ii) We compared genetic distances based on the intron sequences (excluding intronic ORFs) and the 3rd position of the exon sequences. Assuming that accumulation of mutations at these positions is neutral, we expect to find similar amounts of variation at intron and exon sequences if they co-evolved over a long time. In contrast, if the introns were only recently acquired, we expect them to be more similar to each other than the exon sequences. Finally, we tested whether intron gains or losses occurred once or several times independently. In a first BEAST analysis, all species lacking *cox2* introns were constrained to form a monophyletic cluster; i.e. assuming that they all had the same origin. In the second analysis all species possessing *cox2* introns were constrained to form a monophyletic cluster, reflecting the hypothesis of a single origin of the introns. The TRACER v1.5 program (Drummond and Rambaut, 2007) was used to combine the BEAST output log-files and to compare the likelihoods of the resulting constrained and unconstrained trees from the two scenarios using Bayes factors against the results obtained from the unconstrained tree.

3. Results

3.1. Genome content and genome organization

The mt genomes of *Rhynchosporium* spp. are circular molecules of 68,729 bp (*R. secalis*), 69,581 bp (*R. commune*), 68,904 bp (*R. agropyri*) and 49,539 bp (*R. orthosporum*). These sequences were deposited in GenBank (accession numbers KF650575, KF650573, KF650572 and KF650574, respectively) (Figs. 1 and 2A). The overall A + T content was similar for the four mt genomes (Fig. 2A). However intraspecific A and T contents differed at $P < 0.01$ between exon sequences found in the core protein-encoding genes (A-mean = 0.309; T-mean = 0.400) and both introns (A-mean = 0.372; T-mean = 0.335) and free-standing uORFs (A-mean = 0.361; T-mean = 0.331). The mt genomes of the four *Rhynchosporium* spp. had frequencies of perfect microsatellite repeats similar to *P. subalpina* (3.9%), ranging from 3.5% to 4.5% of the total mtDNA. All four mt genomes contained 13 of the standard genes encoding oxidative phosphorylation (Table 1). *Atp9* was present but likely not functional due to a premature stop codon in a conserved domain.

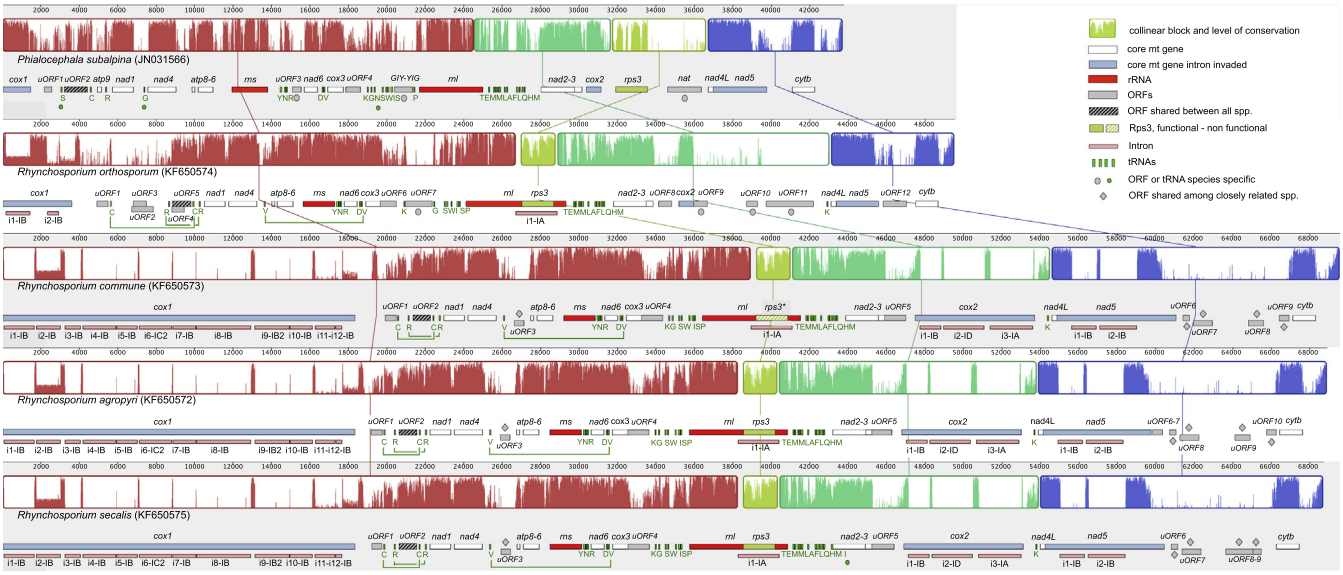
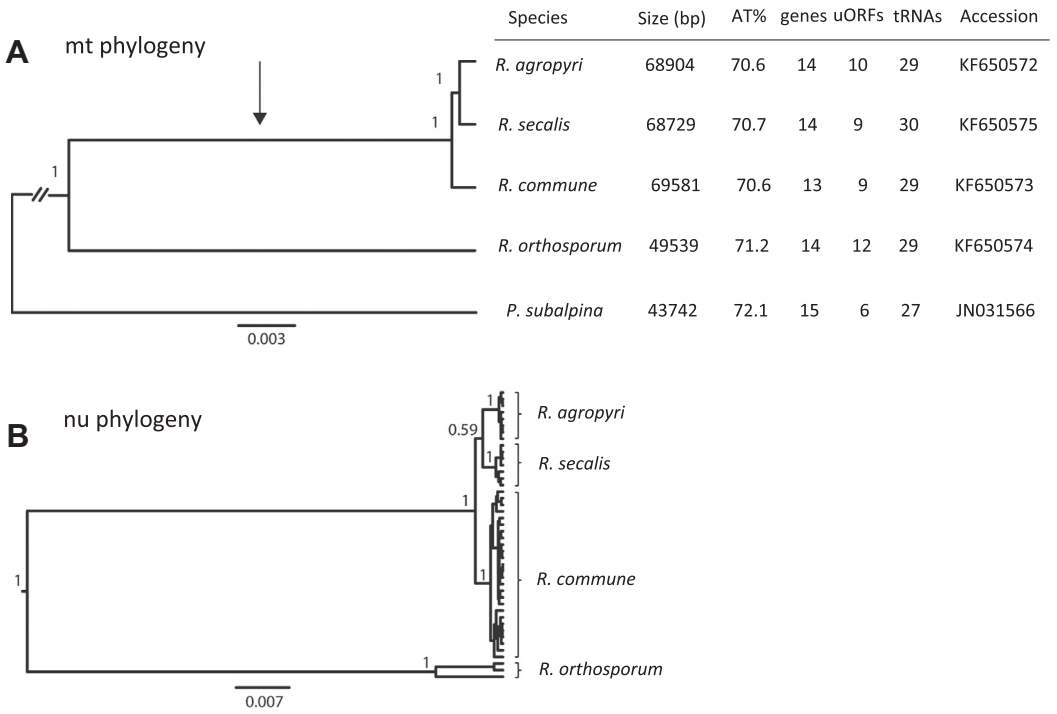


Fig. 1. Linearized maps of the mt genomes of *Rhynchosporium secalis* (NCBI-KF650575), *R. agropyri* (NCBI-KF650572), *R. commune* (NCBI-KF650573) and *R. orthosporum* (NCBI-KF650574) compared to the annotated mt genome of *Phialocephala subalpina* (NJ031566). The *cox1* gene was used as an anchor to linearize the genomes. White, blue, red and gray horizontal bars show respectively core mt genes, core genes invaded by introns, rRNAs and open reading frames of unknown function (uORFs). Pink horizontal bars represent introns and green vertical bars are tRNA genes. Lines connecting tRNAs indicate duplicated tRNAs. Four locally collinear blocks (LCBs) were detected by MAUVE genome alignments. The average level of conservation corresponds to the height of the similarity profile inside each LCB. The only rearranged gene is *rps3*, shown as a light green bar. Gray and green dots show species-specific ORFs and tRNAs. Gray diamonds are ORFs shared by the three closely related *Rhynchosporium* spp., whose sequence could be identified in the other two species. Further details about the mt genome of *P. subalpina* are given in Duò et al. (2012). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Species	Size (bp)	AT%	genes	uORFs	tRNAs	Accession
R. agropyri	68904	70.6	14	10	29	KF650572
R. secalis	68729	70.7	14	9	30	KF650575
R. commune	69581	70.6	13	9	29	KF650573
R. orthosporum	49539	71.2	14	12	29	KF650574
P. subalpina	43742	72.1	15	6	27	JN031566

Fig. 2. (A) Phylogeny based on mtDNA sequences. The bootstrap consensus tree was inferred from 1000 replicates. *Phialocephala subalpina* was used as the outgroup. A discrete Gamma distribution was used to model evolutionary rate differences among sites 5 categories (+G, parameter = 1.4367). The Maximum Likelihood tree is drawn to scale, with branch lengths corresponding to the number of substitutions per site. Mt genome size, AT content, number of genes, unknown open reading frames (uORFs) and tRNAs are shown for each species. The NCBI accession number corresponds to each sequence. The arrow indicates where the intron invasion occurred. (B) Nuclear phylogeny was reconstructed using the concatenated alpha-tubulin, beta-tubulin and ITS sequences described previously by Zaffarano et al. (2008).

The ribosomal protein S3 (*rps3*) was annotated in all *Rhynchosporium* spp. but showed a single nucleotide indel (A245-) that introduced a premature stop codon in *R. commune*. The small (*rns*) and large (*rnl*) ribosomal subunits as well as a complete set of twenty-nine tRNA genes were identified in all mtDNAs (Fig. 1). *R. secalis* had an additional tRNA isoacceptor for isoleucine (tRNA-I) positioned next to *nad2* that was not functional in the other mt genomes (Fig. 1). Three tRNA genes (tRNA-C, tRNA-R and tRNA-

V) were duplicated in the *Rhynchosporium* spp. (Fig. 1). Two tRNA genes (tRNA-S and tRNA-G) present in the outgroup *P. subalpina* were not found in *Rhynchosporium* spp., and tRNA-K was not found in *P. subalpina*. As in other fungal mt genomes, several tRNA genes flanked *rnl*. In the Leotiales species belonging to *PAC* and *Rhynchosporium* the 5′-upstream tRNA gene order consensus (KGSWISP) was generally similar to that of Eurotiomycetes (KGDSWISP). But the 3′-downstream tRNA gene synteny (TEMMLAFLQHM) mostly resembled that of Sordariomycetes (Ghikas et al., 2006; Torriani et al., 2008). All core protein-encoding mt genes were transcribed in the same direction and had the canonical translation initiation codon (ATG), except for *atp6* of *R. orthosporum* (TTG). A single nucleotide indel in a poly-G region of *nad5* caused a premature stop codon in *R. agropyri*. But this premature stop codon did not affect the conserved domains; hence the protein is expected to maintain its functionality. A rare alternative stop codon (CAA) was found for *nad3* in all *Rhynchosporium* spp.

Variable numbers of free-standing uORFs were found in the mt genome of the four *Rhynchosporium* spp., ranging from 12 uORFs in *R. orthosporum* to 9 uORFs in *R. secalis* and *R. commune* (Figs. 1 and 2A). *R. orthosporum* had four unique uORFs with no sequence similarity to other mt genomes. Especially interesting was the largest uORF, *Ro_uORF11*, found in *R. orthosporum*, which encodes an 807 aa protein with five transmembrane domains, but lacks similarity to any protein sequence in the NCBI database, so a function could not be assigned. The nine uORFs conserved in at least two of the three most closely related *Rhynchosporium* species were called "common ORFs" (cORFs, Supplementary Table 1). Only *cORF2* was shared by all four *Rhynchosporium* spp. with a sequence identity of >90%. This ORF showed high amino acid similarity (*E*-value < e^{-44} and identity >54%) to *ORF_02* found in all PAC species (Duò et al., 2012). Based on the TMHMM2 method (Krogh et al., 2001) two additional cORFs (*cORF6* and *cORF1*) were predicted to code for transmembrane proteins. *cORF8* was highly variable in size (from 1536 bp in *R. secalis* to 756 bp in *R. agropyri* and *R. commune*) and it was the least conserved ORF. Sequences for *cORF2*, *cORF4*, *cORF7* and for the overlapping region of *cORF5* and *cORF6* were identified in an *R. commune* EST library (W. Knogge, unpublished). Thus, at least 5 of the 11 predicted uORFs were transcribed, all of them being cORFs.

3.2. Intron invasion and putative horizontal transfers

Three intron presence/absence polymorphisms (IPAPs) in *cox1* (2 IPAPs) and the *rnl* (1 IPAP) distinguished *R. orthosporum* from *P. subalpina*, the closest relative with a completely sequenced mt genome (Fig. 1). The mt genomes of *R. commune*, *R. agropyri* and *R. secalis* were invaded by introns after the split from *R. orthosporum*. If compared to *R. orthosporum* the three genes showing IPAPs were *cox1* (10 IPAPs), *cox2* (3 IPAPs) and *nad5* (2 IPAPs), with an average intron content of 16,573 bp, 5449 bp and 3220 bp, respectively (Fig. 1). This represents an average intron gain compared to *R. orthosporum* of 23,393 bp. *Cox1* introns encoded 8 intronic ORFs of unknown function, 8 ORFs with homology to LAGLIDADG endonucleases and one ORF with homology to GIY endonucleases (Supplementary Fig. 1). *Nad5* introns had two LAGLIDADG endonucleases and an intronic ORF of unknown function (Table 2). *Cox2* introns encoded two GIY endonucleases, an intronic ORF of unknown function and a LAGLIDADG endonuclease.

The maximum-likelihood phylogenetic reconstruction of the unconstrained tree based on *cox2* exon sequences is shown in Supplementary Fig. 2A. Assuming a common ancestor with all three introns (+++) would require 18 subsequent losses to explain the observed IPAPs for each species. In contrast, only five intron gains are necessary if the ancestral mt genome lacked the introns. The average pairwise genetic distance between introns was significantly smaller than the distance between corresponding exons (Supplementary Fig. 3B). All of these results are consistent with a more recent phylogenetic history for the introns, supporting the hypothesis of intron gains.

Six introns (*cox1*_i2, i5, i7, i10, i11 and cox2_i1) showed evidence of putative horizontal transfer, with total nucleotide query cover values >90%, intron sequence identity >79% and *E*-values = 0. Four introns (*cox1*_i4, i8, i9, i12) had a similar nucleotide identity, but much lower query cover values (36–70%), suggesting that indel mutations occurred after the putative horizontal transfer. Only three of the 12 *cox1* introns (*cox1*_i1, i3, i6) did not show homology to other fungal introns. The introns with highest homology, *cox1*_i2 (100% Q-cover, 91% nucleotide identity) and *cox1*_i11 (99% Q-cover, 95% nucleotide identity), were homologous to mt introns of *Marssonina brunnea*, the causal agent of Marssonina leaf spot, one of the most damaging foliage diseases of poplar. The recently sequenced *M. brunnea* (Zhu et al., 2012) belongs to the Leotiomycetes, the same ascomycete class as *Rhynchosporium* and PAC spp. Three additional introns (*cox1*_i4, i8, i12) shared homologies only with *M. brunnea* introns. Three introns showed strong homology to more than one species (*cox1*_i5, i10 and *cox2*_i1). *Cox1*_i10 was the most widely distributed intron among the fungal species deposited in the NCBI database, with at least seven species having a related intron. Eight of the eleven LAGLIDADG ORFs and two of the three GIY-YIG ORFs from *Rhynchosporium* spp. had high homology to intron-encoded endonucleases from other fungal species. None of the annotated iORFs of unknown function shared similarity (Table 2).

Table 1
Comparison of core mt genes. Four *Rhynchosporium* spp. were compared to identify synonymous, non-synonymous and indel mutations. Nucleotide diversity (Pi) and sequence conservation (SC), the proportion of conserved sites in the alignment, were measured using the program DnaSP v5.10.1 (Librado and Rozas, 2009).

Gene	Sites (bp)[a]	SNPs	Syn	Non Syn	Indel (bp)	Pi	SC
atp6	819	26	19	7	1 (3)	0.0159	0.968
atp8	147	8	7	1	0	0.0272	0.946
cox1	1752	199	140	59	0	0.0583	0.885
cox2	756	53	46	7	0	0.0355	0.93
cox3	810	28	25	3	0	0.0173	0.965
cytb	1161	54	44	10	0	0.0235	0.953
nad1	1083	29	26	3	0	0.0134	0.973
nad2	1686	66	43	23	1 (6)	0.0196	0.961
nad3	354	27	27	0	0	0.0381	0.924
nad4	1464	36	28	8	0	0.0123	0.975
nad4L	270	6	6	0	0	0.0111	0.978
nad5[b]	1962	69	57	12	0	0.0175	0.965
nad6	675	15	12	3	0	0.0111	0.978

[a] Total number of sites (excluding sites with gaps or missing data).
[b] Only partial sequence analyzed, because of the very variable terminal part.

3.3. Mutation rate estimates and inferred phylogeny

Using the average TMRCA of 26,853 ybp inferred from nuDNA variation as a calibration point (Zaffarano et al., 2008) we obtained a mean rate for mtDNA evolution of 6.80E−07 substitution/site/year. Based on this estimate, the mt genome showed a 77-fold higher mutation rate than the nu genome (95% HPD, 22–1250-fold higher).

The inferred mt phylogenetic relationship resulted in a tree with maximum posterior probabilities for all nodes (Fig. 2A). *R. agropyri* was inferred to be the closest sister species of *R. secalis* (posterior probability = 1), providing the best fit to the mt data by Bayes scores when testing all possible combinations of sister species (Supplementary Fig. 3A). In contrast, the phylogeny inferred using nu markers resulted in an ambiguous topology with regard to the positioning of *R. secalis*. The earlier analysis con-

Table 2
Intron-encoded ORF similarities. *E*-value (expected value) and Id (maximal identity) from database searches using the NCBI BLASTN algorithm and *Rhynchosporium commune* sequences as query. Associated accession numbers from NCBI are listed. iORFs were further analyzed using the NCBI BLASTP algorithm without finding similarities with *E*-values < 1E−5.

Gene	Intron	ORF	*E*-value	Id	Similarity	Accession
cox1	Intron 1	iORF	–	–	–	–
		LAGLIDADG	–	–	–	–
	Intron 2	LAGLIDADG	0	92%	*Marssonina brunnea*	JN204424.1
	Intron 3	iORF	–	–	–	–
	Intron 4	LAGLIDADG	0	89%	*Marssonina brunnea*	JN204424.1
		iORF	–	–	–	–
	Intron 5	LAGLIDADG	3E−167	87%	*Neurospora crassa*	X14669.1
			6E−159	86%	*Leptosphaeria maculan*	NW_003533854.1
			3E−48	75%	*Gibberella zeae*	DQ364632.1
	Intron 6	iORF	–	–	–	–
	Intron 7	LAGLIDADG	0	80%	*Peltigera malacea*	JN088164.1
			2E−52	92%	*Macrophomina phaseolina*	GU944277.1
	Intron 8	LAGLIDADG	0	92%	*Marssonina brunnea*	JN204424.1
		iORF	–	–	–	–
		iORF	–	–	–	–
		iORF	–	–	–	–
	Intron 9	LAGLIDADG	5E−152	81%	*Podospora anserina*	X55026.1
		iORF	–	–	–	–
	Intron 10	LAGLIDADG	0	82%	*Ajellomyces dermatitidis*	XM_002620019.1
			0	81%	*Peltigera malacea*	JN088164.1
			0	81%	*Ceratocystis cacaofunesta*	JX185564.1
			4E−163	78%	*Hypocrea jecorina*	AF447590.1
			2E−51	75%	*Aspergillus kawachii*	AP012272.1
			1E−48	74%	*Aspergillus tubingensis*	AY802759.1
			9E−46	74%	*Fusarium solani*	JN041209.1
	Intron 11	GIY-YIG	0	94%	*Marssonina brunnea*	JN204424.1
	Intron 12	–	–	–	–	–
cox2	Intron 1	GIY-YIG	2E−125	78%	*Podospora anserina*	X55026.1
			8E−35	80%	*Sordaria macrospora*	XM_003342359.1
	Intron 2	LAGLIDADG	4E−64	77%	*Hypocrea jecorina*	AF447590.1
			4E−64	77%	*Trichoderma reesei*	Y14564.1
			2E−41	74%	*Leptosphaeria maculans*	NW_003533854.1
		iORF	–	–	–	–
	Intron 3	GIY-YIG	–	–	–	–
nad5	Intron 1	LAGLIDADG	–	–	–	–
	Intron 2	LAGLIDADG	–	–	–	–
		iORF	–	–	–	–

ducted by Zaffarano et al. (2008) identified *R. secalis* as being more closely related to *R. commune*. In our analysis *R. secalis* paired with *R. agropyri* in concordance with the mt data set, however, with a non-significant statistical support (posterior = 0.59; Fig. 2B). This unresolved topology in the nu tree is also reflected in the tests involving different sister species combinations. The combinations *R. secalis*/*R. agropyri* and *R. agropyri*/*R. commune* gave very similar scores, i.e. they were equally likely (Supplementary Fig. 3B). The first intron acquisition of *cox1* occurred before the split of *R. orthosporum* and the other species. The intron invasion of *cox1*, *cox2* and *nad5* happened afterward.

4. Discussion

The large increase in mt genome size for *R. secalis*, *R. commune* and *R. agropyri*, compared to *R. orthosporum*, is due mainly to an invasion of introns into *cox1*, *cox2* and *nad5*. Variation in mt genome size among *Rhynchosporium* spp. is affected mainly by differences in the numbers of introns with minor differences in the length and organization of intergenic regions, including differences in the number of uORFs (Fig. 2A). Mt genomes of fungi and plants often possess different numbers of introns that vary in size from 0.15 to 4 kb (Burger et al., 2003) and overall reported intron content ranges from 0% in *Aspergillus terreus* and *Z. tritici* to 75% in *P. anserina* (Cummings et al., 1990; Joardar et al., 2012; Torriani et al., 2008). In the *Rhynchosporium* clade, intron content increased from 8.1% in *R. orthosporum* to 39.8% in both *R. agropyri* and *R. secalis*. Mt introns are highly invasive, but little is known about the evolutionary time needed to fix these genetic elements because few comparisons have been made among closely related species. Self-splicing introns and related homing endonucleases were defined as parasitic genetic elements showing super-Mendelian inheritance, which allows them to become fixed in populations despite not providing a fitness advantage for the host species (Goddard and Burt, 1999; Gogarten and Hilario, 2006). The interspecific comparison with the closely related PAC species did not provide information relevant to intron evolution in these mt genomes because the PAC species carry very few introns (Duò et al., 2012). The posterior mean estimates of TMRCA for all *Rhynchosporium* spp. were between ~14,000–35,000 years B.P. (95% HPD, ~1000–102,000) (Zaffarano et al., 2008). This suggests that a maximum of ~100,000 years elapsed between the split of the three youngest *Rhynchosporium* spp. from *R. orthosporum* and the introgression of 15 introns into their mt genomes. The most parsimonious explanation for this rapid and recent intron invasion is horizontal transfer from donor species. Our indirect attempt to quantify the evolutionary time needed for intron horizontal transmission is consistent with a much shorter time than the previously report of 10^6–10^7 years obtained by comparing 20 species of yeasts (Goddard and Burt, 1999).

A recent analysis of 640 angiosperms from 212 families indicated that introns were acquired through approximately 70 distinct horizontal transfer events (Sanchez-Puerta et al., 2008). This horizontal transfer of introns among mt genomes of flowering

plants was dominated by exchanges among plants from the same family and illegitimate pollination was proposed as the most likely mechanism for the lateral transfer (Sanchez-Puerta et al., 2008). The presence of *cox2* introns in *P. anserina* and *Gibberella zeae* in the unconstrained phylogenetic tree (Supplementary Fig. 2A) suggests at least three independent intron introductions. This conclusion was supported by a higher log-likelihood distribution, and thus greater support, for the unconstrained tree topology (mean = −3973) over the constrained tree representing monophyly of the intron introduction (mean = −4205). This difference is highly significant according to the Bayes Factor comparison, log10BF = 11.8, meaning that the multiple origin model is $10^{11.8}$ times more likely than the model of a single monophyletic origin (Supplementary Fig. 4). Two fungal species can directly exchange genetic material through mycelial or conidial anastomoses if they share the same ecological niche (Hane et al., 2011; Roca and Read, 2005). Another possibility is indirect transmission of genetic material through a broad host range virus that can move DNA or RNA from one fungal species to the other (Syvanen, 2012). The identification of the donor species is difficult, however, because introns and their iORFs might have different origins (Mota and Collins, 1988; Sellem and Belcour, 1997). As reported earlier for flowering plants, intron invasion of fungal mt genes may be accompanied by co-conversion of flanking exonic regions (Sanchez-Puerta et al., 2008). Co-conversion of flanking exonic regions in *Rhynchosporium* spp. affected mainly 5–10 bp of the 5′ and 3′ exons (Fig. 3), but it was not found for all intron–exon boundaries.

Atp9 and *rps3* are the only two protein-coding genes among the 15 inferred to exist in the ancestral fungal mt genome that have been lost or transferred to the nucleus (Adams and Palmer, 2003). A comparative analysis of 26 fungal species found high levels of diversity for *atp9* in mt genomes (Déquard-Chablat et al., 2011). None of the *Rhynchosporium* mt genomes carried a functional copy of *atp9*, but a region showing high nucleotide similarity to the *atp9* of *P. subalpina* was found in all *Rhynchosporium* spp. (76.6% identity to GenBank JN031566 (Duò et al., 2012)). As expected, *rps3* is located within an intron in the U11 region of *rnl* (Gibb and Hausner, 2005; Sethuraman et al., 2009). A single nucleotide indel (A245-) in *rps3* of *R. commune* introduces a premature stop codon, thus *rps3* may not function in *R. commune*. Free-standing *rps3* genes in mt genomes have been reported only twice among the sequenced filamentous ascomycetes (Duò et al., 2012; Hane et al., 2007). *P. subalpina*, the closest sequenced relative of *Rhynchosporium* spp., has a free-standing *rps3* gene located next to *cox2*. *Rps3* was the only gene lacking synteny between PAC and *Rhynchosporium* spp. (Fig. 1). *P. subalpina* also has a unique putative N-acetyl-transferase gene next to *rps3*. Since the N-acetyl-transferase gene of *P. subalpina* is unique in the mt genomes of ascomycetes (Duò et al., 2012), it is likely that this gene represents an independent gene acquisition in PAC species rather than having been lost from *Rhynchosporium* spp.

uORFs are often annotated in fungal mt genomes (Cummings et al., 1990; Duò et al., 2012; Hane et al., 2007; Torriani et al., 2008), but their origins and functions have not yet been elucidated. It is highly unusual to find mt uORFs shared between distantly related species. cORF2 was found in 12 fungal species belonging to PAC (Duò et al., 2012) and *Rhynchosporium* spp. (Fig. 1) and showed a degree of conservation similar to the core mt genes (Supplementary Tables 1 and 2). *R. commune*, *R. agropyri*, and *R. secalis* share several uORFs with high homology, consistent with a very recent divergence of these sister species. A Bayesian analysis of divergence time estimated that these three lineages emerged between 1200 and 3600 years ago (Zaffarano et al., 2008). While this short evolutionary timeframe was not sufficient to introduce many mutations, small rearrangements were found, especially between *nad5* and *cytb*, e.g. *cORF9* was not found in *R. secalis*.

We consider three mechanisms as being most likely to explain the origin of uORFs in mt genomes. The first mechanism involves

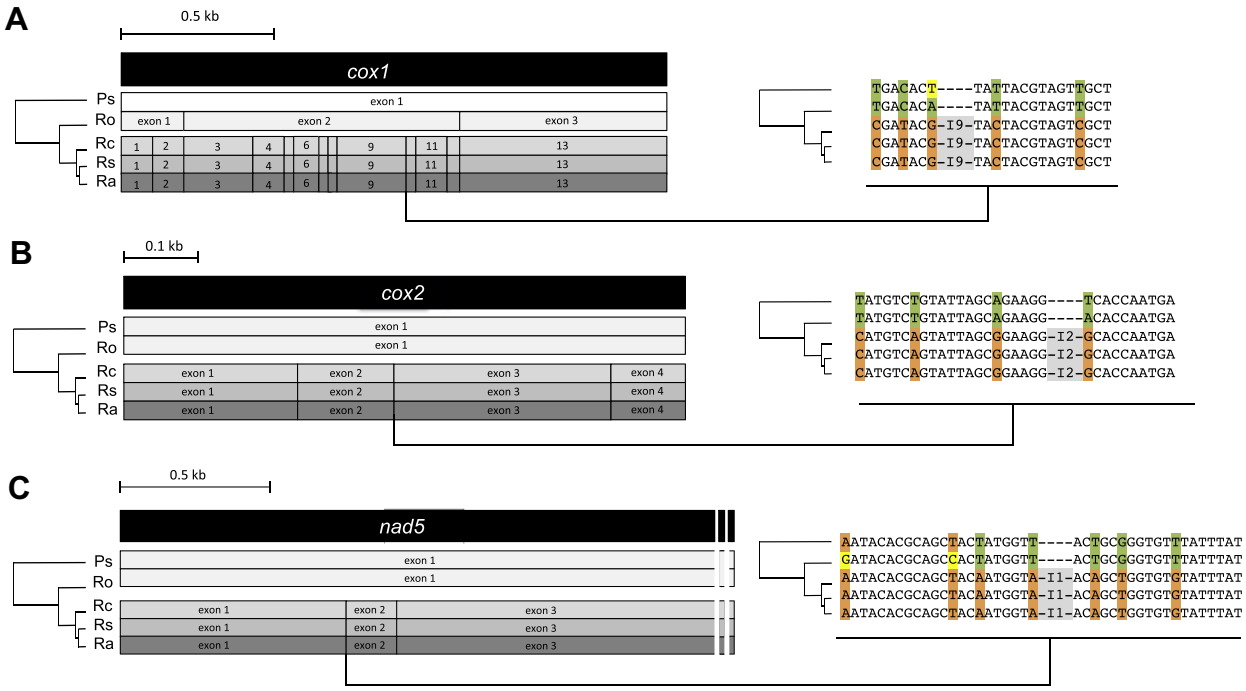


Fig. 3. Structures of the three intron-containing genes of *Rhynchosporium* spp. (Ps = *P. subalpina*, Ro = *R. orthosporum*, Rc = *R. commune*, Rs = *R. secalis*, Ra = *R. agropyri*). The outgroup Ps does not contain introns. For each species exon numbers are reported and vertical lines represent intron insertion points. One example of co-conversion of the flanking exonic regions is presented for each gene (intron 9 of *cox1* (A); intron 2 of *cox2* (B); and intron 1 of *nad5* (C)). Co-conversion mutation sites are green in species lacking introns and orange in species carrying the introns. Yellow mutations are unrelated to co-conversion. Not all exon–intron boundaries of *cox1*, *cox2* and *nad5* show co-conversion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the introduction of novel point mutations and small indels into a pre-existing mt gene that disrupts the gene and creates a novel ORF. The second mechanism is transfer of a gene from the nu genome into the mt genome. The third mechanism is horizontal transfer from another species. Additional evidence for the rapid emergence of new uORFs in mt genomes came from comparing mt genome sequences of the close relatives *Z. tritici*, *Zymoseptoria pseudotritici*, *Zymoseptoria ardabiliae*, and *Zymoseptoria passerinii* (SFFT, BAM, and E.H. Stukenbrock, unpublished data). These findings indicate that comparing mt genomes of very closely related fungal species may lead to a better understanding of the mechanisms underlying the origins of uORFs in mt genomes. The presence of varying uORF compositions in the mt genomes of *Rhynchosporium* spp. and other organisms is consistent with a rapidly evolving accessory compartment in the mt genome (Croll and McDonald, 2012). Under this scenario, the slowly evolving core mt genes are highly conserved and adapted to the specific function of oxidative phosphorylation while the rapidly evolving accessory genome may play a supporting role in core mitochondrial function or encode new functions that are not directly connected to oxidative phosphorylation. The accessory compartment of the mt genome in *Rhynchosporium* spp., composed of introns and free standing uORFs, showed an unusual nucleotide composition compared to the core genome, consistent with a recent introduction of these sequences into the mt genome.

It has long been thought that higher mutation rates in mt genomes are unique to animals (Palmer et al., 2000; Wolfe et al., 1987). In vertebrates, the mean ratio of mt to nu base substitutions has been reported as 19:1 and in invertebrates it is 8:1 (Lynch, 2007). However, recent studies challenge this view. For example, long-term mutation accumulation experiments with the yeast *Saccharomyces cerevisiae* using whole-genome sequencing to assay spontaneous mutations estimated the number of mt:nu nucleotide substitutions at 37:1 (Lynch et al., 2008). Our indirect approach based on a relaxed lognormal clock model provided a surprisingly similar estimate of 77:1, consistent with the hypothesis of a much higher substitution rate for the mt genes in fungi. The maximum clade credibility trees based on nu and mt sequences showed identical topologies (Fig. 2A and B). However, the statistical support for the sister species status of *R. agropyri* and *R. secalis* was higher in the phylogeny based on the mt genome compared to the nu genome sequences. This observation was supported by subsequent tests involving different sister species combinations (Supplementary Fig. 3A and B).

Genes in mt genomes have long been the marker of choice for biogeographic and phylogenetic studies, especially in vertebrates (Avise et al., 1987). This wide application was due mainly to the relatively easy identification of orthologous gene sequences coupled with little or no recombination and rapid evolution compared to nu genes. It was also assumed that mt genes evolve as neutral markers, making them particularly suitable for ecological and evolutionary studies (Ballard and Kreitman, 1995). However, this view was challenged (Parmakelis et al., 2013; Rand, 2001).

The fungal mitochondrial genome project (FMGP) sequenced the complete mt genomes of a small number of distantly related species representing the major fungal lineages to compare genome structure, gene content and the evolution of gene expression over the course of fungal evolution (Paquin et al., 1997). As a result of the rapidly decreasing costs in sequencing mt genomes, it has become practical to compare mt genomes of closely related fungal species. These new comparisons place mt genome evolution into a more recent evolutionary context and enable a better comparison of the tempo and mode of evolution in mt genomes compared to nu genomes. Our study revealed a very dynamic genome evolution, challenging the view of mt genome sequences evolving neutrally. We showed that fungal mt genomes evolve very rapidly through a combination of processes, including horizontal transfer and intron invasion. Our findings show that studies based on mt genomes can provide novel insights into recently diverged fungal complexes as a result of their rapid evolution. The likely non-neutral evolution of the fungal mt genome provides both challenges and opportunities to study fundamental mitochondrial processes, although the role and function of the rapidly evolving accessory compartment remains a mystery.

Acknowledgments

The authors wish to thank the Leibniz Association for financial support in the framework of the Pact for Research and Innovation. A portion of the research was supported by a grant from ETH Zurich (TH-02 07-1).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.11.001.

References

Adams, K.L., Palmer, J.D., 2003. Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol. Phylogenet. Evol. 29, 380–395.
Adams, K.L., Daley, D.O., Qiu, Y.L., Whelan, J., Palmer, J.D., 2000. Repeated, recent and diverse transfers of a mitochondrial gene to the nucleus in flowering plants. Nature 408, 354–357.
Al-Reedy, R.M., Malireddy, R., Dillman, C.B., 2012. Comparative analysis of *Fusarium* mitochondrial genomes reveals a highly variable region that encodes an exceptionally large open reading frame. Fungal Genet. Biol. 49, 2–14.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. 18, 489–522.
Ballard, J.W., Kreitman, M., 1995. Is mitochondrial DNA a strictly neutral marker? Trends Ecol. Evol. 10, 485–488.
Beaudet, D., Nadimi, M., Iffis, B., Hijri, M., 2013. Rapid mitochondrial genome evolution through invasion of mobile elements in two closely related species of arbuscular mycorrhizal fungi. PLoS One 8, e60768.
Burger, G., Gray, M.W., Lang, B.F., 2003. Mitochondrial genomes: anything goes. Trends Genet. 19, 709–716.
Caldwell, R.M., 1937. *Rhynchosporium* scald of Barley, Rye, and other grasses. J. Agric. Res. 55, 175–198.
Campbell, A., Mrázek, J., Karlin, S., 1999. Genome signature comparisons among prokaryote, plasmid, and mitochondrial DNA. Proc. Natl. Acad. Sci. USA 96, 9184–9189.
Chen, J.Z., Hebert, P.D., 1999. Intraindividual sequence diversity and a hierarchical approach to the study of mitochondrial DNA mutations. Mutat. Res. 434, 205–217.
Croll, D., McDonald, B.A., 2012. The accessory genome as a cradle for adaptive evolution in pathogens. PLoS Pathog. 8, e1002608.
Cummings, D.J., McNally, K.L., Domenico, J.M., Matsuura, E.T., 1990. The complete DNA sequence of the mitochondrial genome of *Podospora anserina*. Curr. Genet. 17, 375–402.
Déquard-Chablat, M., Sellem, C.H., Golik, P., Bidard, F., Martos, A., Bietenhader, M., di Rago, J.-P., Sainsard-Chanet, A., Hermann-Le Denmat, S., Contamine, V., 2011. Two nuclear life cycle-regulated genes encode interchangeable subunits c of mitochondrial ATP synthase in *Podospora anserina*. Mol. Biol. Evol. 28, 2063–2075.
Drummond, A.J., Rambaut, A., Shapiro, B., Pybus, O.G., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol. Biol. Evol. 22, 1185–1192.
Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.
Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.5.
Duò, A., Bruggmann, R., Zoller, S., Bernt, M., Grunig, C.R., 2012. Mitochondrial genome evolution in species belonging to the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex. BMC Genomics 13, 166.
Ghikas, D.V., Kouvelis, V.N., Typas, M.A., 2006. The complete mitochondrial genome of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*: gene order and trn gene clusters reveal a common evolutionary course for all Sordariomycetes, while intergenic regions show variation. Arch. Microbiol. 185, 393–401.
Ghikas, D.V., Kouvelis, V.N., Typas, M.A., 2010. Phylogenetic and biogeographic implications inferred by mitochondrial intergenic region analyses and ITS1-

5.8S-ITS2 of the entomopathogenic fungi *Beauveria bassiana* and *B. brongniartii*. BMC Microbiol. 10, 174.
Gibb, E.A., Hausner, G., 2005. Optional mitochondrial introns and evidence for a homing-endonuclease gene in the mtDNA rnl gene in *Ophiostoma ulmi* s. lat. Mycol. Res. 109, 1112–1126.
Goddard, M.R., Burt, A., 1999. Recurrent invasion and extinction of a selfish gene. Proc. Natl. Acad. Sci. USA 96, 13880–13885.
Gogarten, J.P., Hilario, E., 2006. Inteins, introns, and homing endonucleases: recent revelations about the life cycle of parasitic genetic elements. BMC Evol. Biol. 6, 94.
Goodwin, S., 2002. The barley scald pathogen *Rhynchosporium secalis* is closely related to the discomycetes Tapesia and Pyrenopeziza. Mycol. Res. 106, 645–654.
Gray, M.W., 1999. Mitochondrial evolution. Science 283, 1476–1481.
Hane, J.K., Lowe, R.G.T., Solomon, P.S., Tan, K.-C., Schoch, C.L., Spatafora, J.W., Crous, P.W., Kodira, C., Birren, B.W., Galagan, J.E., Torriani, S.F.F., McDonald, B.A., Oliver, R.P., 2007. Dothideomycete–plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. Plant Cell 19, 3347–3368.
Hane, J.K., Rouxel, T., Howlett, B.J., Kema, G.H., Goodwin, S.B., Oliver, R.P., 2011. A novel mode of chromosomal evolution peculiar to filamentous Ascomycete fungi. Genome Biol. 12, R45.
Herrmann, J.M., 2003. Converting bacteria to organelles: evolution of mitochondrial protein sorting. Trends Microbiol. 11, 74–79.
Jacobs, M.A., Payne, S.R., Bendich, A.J., 1996. Moving pictures and pulsed-field gel electrophoresis show only linear mitochondrial DNA molecules from yeasts with linear-mapping and circular-mapping mitochondrial genomes. Curr. Genet. 30, 3–11.
Joardar, V., Abrams, N.F., Hostetler, J., Paukstelis, P.J., Pakala, S., Pakala, S.B., Zafar, N., Abolude, O.O., Payne, G., Andrianopoulos, A., Denning, D.W., Nierman, W.C., 2012. Sequencing of mitochondrial genomes of nine Aspergillus and Penicillium species identifies mobile introns and accessory genes as main sources of genome size variability. BMC Genomics 13, 698.
John, P., Whatley, F.R., 1975. *Paracoccus denitrificans* and the evolutionary origin of the mitochondrion. Nature 254, 495–498.
Jurica, M.S., Stoddard, B.L., 1999. Homing endonucleases: structure, function and evolution. CMLS. Cell. Mol. Life Sci. 55, 1304–1326.
Kasuga, T.T., White, T.J.T., Taylor, J.W.J., 2002. Estimation of nucleotide substitution rates in Eurotiomycete fungi. Mol. Biol. Evol. 19, 2318–2324.
Krogh, A., Larsson, B.È., Heijne, Von, G., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567–580.
Lang, B.F., Gray, M.W., Burger, G., 1999. Mitochondrial genome evolution and the origin of eukaryotes. Annu. Rev. Genet. 33, 351–397.
Lang, B.F., Laforest, M.-J., Burger, G., 2007. Mitochondrial introns: a critical view. Trends Genet. 23, 119–125.
Lehnackers, H., Knogge, W., 1990. Cytological studies on the infection of barley cultivars with known resistance genotypes by *Rhynchosporium secalis*. Can. J. Botany 68, 1953–1961.
Li, W., Tucker, A.E., Sung, W., Thomas, W.K., Lynch, M., 2009. Extensive, recent intron gains in Daphnia populations. Science 326, 1260–1262.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Lowe, T.M., Eddy, S.R., 1997. TRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964.
Lynch, M., 2007. The Origins of Genome Architecture. Sinauer Associates Inc..
Lynch, M., Sung, W., Morris, K., Coffey, N., Landry, C.R., Dopman, E.B., Dickinson, W.J., Okamoto, K., Kulkarni, S., Hartl, D.L., Thomas, W.K., 2008. A genome-wide view of the spectrum of spontaneous mutations in yeast. Proc. Natl. Acad. Sci. USA 105, 9272–9277.
Maleszka, R., Clark-Walker, G.D., 1992. In vivo conformation of mitochondrial DNA in fungi and zoosporic moulds. Curr. Genet. 22, 341–344.
Moore, W.S., 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution 49, 718–726.
Mota, E.M., Collins, R.A., 1988. Independent evolution of structural and coding regions in a Neurospora mitochondrial intron. Nature 332, 654–656.
Palmer, J.D., Adams, K.L., Cho, Y., Parkinson, C.L., Qiu, Y.L., Song, K., 2000. Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. Proc. Natl. Acad. Sci. USA 97, 6960–6966.
Paquin, B., Laforest, M.J., Forget, L., Roewer, I., Wang, Z., Longcore, J., Lang, B.F., 1997. The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. Curr. Genet. 31, 380–395.
Parmakelis, A., Kotsakiozi, P., Rand, D., 2013. Animal mitochondria, positive selection and cyto-nuclear coevolution: insights from pulmonates. PLoS One 8, e61970.
Rand, D.M., 2001. The units of selection on mitochondrial DNA. Annu. Rev. Ecol. Syst. 32, 415–448.
Roca, M.G., Read, N.D., 2005. Conidial anastomosis tubes in filamentous fungi – Gabriela Roca – 2006 – FEMS Microb. Let. – Wiley Online Library. FEMS Microbiol. Lett. 249, 191–199.
Rohe, M., Searle, J., Newton, A.C., Knogge, W., 1996. Transformation of the plant pathogenic fungus, *Rhynchosporium secalis*. Curr. Genet. 29, 587–590.
Sanchez-Puerta, M.V., Cho, Y., Mower, J.P., Alverson, A.J., Palmer, J.D., 2008. Frequent, phylogenetically local horizontal transfer of the *cox1* Group I intron in flowering plant mitochondria. Mol. Biol. Evol. 25, 1762–1777.
Saraste, M., 1999. Oxidative phosphorylation at the fin de siècle. Science 283, 1488–1493.
Sellem, C.H., Belcour, L., 1997. Intron open reading frames as mobile elements and evolution of a group I intron. Mol. Biol. Evol. 14, 518–526.
Sethuraman, J., Majer, A., Friedrich, N.C., Edgell, D.R., Hausner, G., 2009. Genes within Genes: multiple LAGLIDADG homing endonucleases target the ribosomal protein S3 gene encoded within an *rnl* group I intron of Ophiostoma and related taxa. Mol. Biol. Evol. 26, 2299–2315.
Shipton, W., Boyd, W.J.R., Ali, S.M., 1974. Scald of barley. Rev. Plant Pathol. 53, 839–861.
Syvanen, M., 2012. Evolutionary implications of horizontal gene transfer. Annu. Rev. Genet. 46, 341–358.
Torriani, S.F.F., Goodwin, S.B., Kema, G.H.J., Pangilinan, J.L., McDonald, B.A., 2008. Intraspecific comparison and annotation of two complete mitochondrial genome sequences from the plant pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 45, 628–637.
Torriani, S.F.F., Brunner, P.C., McDonald, B.A., Sierotzki, H., 2009. QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. Pest Manag. Sci. 65, 155–162.
Torriani, S.F.F., Brunner, P.C., McDonald, B.A., Croll, D., 2011. Evidence for extensive recent intron transposition in closely related fungi. Curr. Biol. 21, 2017–2022.
Welty, R., Metzger, R., 1996. First report of scald of triticale caused by *Rhynchosporium secalis* in North America. Plant Dis. 80, 1220–1223.
Wolfe, K.H., Li, W.H., Sharp, P.M., 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc. Natl. Acad. Sci. USA 84, 9054–9058.
Zaffarano, P.L., McDonald, B.A., Linde, C.C., 2008. Rapid speciation following recent host shifts in the plant pathogenic fungus *Rhynchosporium*. Evolution 62, 1418–1436.
Zaffarano, P.L., McDonald, B.A., Linde, C.C., 2009. Phylogeographical analyses reveal global migration patterns of the barley scald pathogen *Rhynchosporium secalis*. Mol. Ecol. 18, 279–293.
Zaffarano, P.L., McDonald, B.A., Linde, C.C., 2011. Two new species of *Rhynchosporium*. Mycologia 103, 195–202.
Zdobnov, E.M., Apweiler, R., 2001. InterProScan an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17, 847–848.
Zhu, S., Cao, Y.Z., Jiang, C., Tan, B.Y., Wang, Z., 2012. Sequencing the genome of *Marssonina brunnea* reveals fungus-poplar co-evolution. BMC Genomics 13, 382.