Multiple Molecular Mechanisms Cause Reproductive Isolation between Three Yeast Species

Jui-Yu Chou1,2, Yin-Shan Hung2, Kuan-Huei Lin1,2, Hsin-Yi Lee2,3, Jun-Yi Leu2*

1 Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan, 2 Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, 3 Molecular Cell Biology, Taiwan International Graduate Program, Graduate Institute of Life Sciences, National Defense Medical Center and Academia Sinica, Taipei, Taiwan

Abstract

Nuclear-mitochondrial conflict (cytonuclear incompatibility) is a specific form of Dobzhansky-Muller incompatibility previously shown to cause reproductive isolation in two yeast species. Here, we identified two new incompatibility genes, MRS1 and AIM22, through a systematic study of F2 hybrid sterility caused by cytonuclear incompatibility in three closely related Saccharomyces species (S. cerevisiae, S. paradoxus, and S. bayanus). MRS1 is a nuclear gene product required for splicing specific introns in the mitochondrial COX1, and Aim22 is a ligase encoded in the nucleus that is required for mitochondrial protein lipoylation. By comparing different species, our result suggests that the functional changes in MRS1 are a result of coevolution with changes in the COX1 introns. Further molecular analyses demonstrate that three nonsynonymous mutations are responsible for the functional differences of Mrs1 between these species. Functional complementation assays to determine when these incompatible genes altered their functions show a strong correlation between the sequence-based phylogeny and the evolution of cytonuclear incompatibility. Our results suggest that nuclear-mitochondrial incompatibility may represent a general mechanism of reproductive isolation during yeast evolution.

Introduction

Reproductive isolation preventing gene flow between diverging populations is crucial for the process of speciation [1]. One of the general reproductive isolation mechanisms that lead to hybrid inviability or sterility is genetic incompatibility (Dobzhansky-Muller incompatibility), which is caused by improper interactions between genetic loci that have functionally diverged in two different species [2,3]. Since genetic incompatibility probably plays an important role at the incipient stage of speciation, identifying the incompatible loci and determining the selection forces underlying their functional divergence are vital for our understanding of how speciation occurs.

In the past two decades, scientists have discovered several genetic loci causing hybrid sterility or inviability [4,5]. Genes involved in genetic incompatibility have been cloned from a variety of organisms, including flies, flatfishes, mice, and Arabidopsis [6,7,8,9,10,11]. Nonetheless, most of the genes identified were from Drosophila, and in most cases, only one component of the incompatible genetic loci was cloned. Systematic studies that involve more than two species in other organisms are still rare. Baker’s yeast, Saccharomyces cerevisiae, and its close relatives, the Saccharomyces sensu stricto yeasts, represent an interesting system for studying genetic incompatibility. These yeasts can mate with each other freely under laboratory conditions. Diploid hybrids collected from the wild or generated in the laboratory can reproduce asexually without showing any obvious defect. However, the viability of hybrid gametes (spores) is very low (about 0.5%–1%), suggesting that there is strong postzygotic reproductive isolation between these yeast species [12]. Because yeast differs from flies in cellular complexity, life style, and population structure, studies in yeast will greatly expand our knowledge of speciation processes [13].

Using chromosome replacement lines of hybrids of two yeast species, a previous study identified a strong incompatibility between a S. bayanus nuclear gene, AEP2, and S. cerevisiae mitochondria that leads to interspecific F2 hybrid sterility [14]. It was found that the 5′-UTR regions of a mitochondrion gene, OLI1, have diverged dramatically between these two species. Since interactions between the Aep2 protein and the 5′-UTR region of the OLI1 mRNA are essential for OLI1 translation, the incompatibility is probably caused by the failure of Sb-Aep2 to recognize the divergent 5′-UTR region of Sc-OLI1. The finding raises a few interesting questions: Does the cytonuclear incompatibility play a general role in yeast reproductive isolation, does the AEP2-OLI1 type of interaction (activation of mRNA translation) represent a common mode of cytonuclear incompatibility, and is there a specific selective force driving this type of cytonuclear evolution?

The mitochondrion is a critical component of cellular energy production and several metabolic pathways. In many organisms including yeast, proper mitochondrial functions are required for gamete development [15,16,17]. The mitochondrion contains its own genome, though one in an advanced state of degeneration.
Most genes essential for mitochondrial functions have been transferred from the proto-mitochondrion genome to that of their host [18,19]. As a consequence of these events, gene products from both mitochondrial and nuclear genomes are required for proper mitochondrial operations [20,21]. In yeast, for example, the mitochondrial genome encodes only eight proteins, but it is estimated that ~1,000 proteins function in mitochondria [22]. Although these two genomes are under different mutation and selection pressures, they are constrained to evolve coordinately to maintain optimal functions [23]; any change in mitochondria (adaptive or drifted) may require one or more consecutive changes in the nucleus [23,24]. This type of interaction provides an ideal background for the evolution of Dobzhansky-Muller incompatibilities; when two populations containing well-adapted cytonuclear genomes coexist, gene products from both mitochondrial and nuclear genomes [32,33]. From these studies, we know that the deleterious effects of cytonuclear incompatibility can lead to reduced fitness, hybrid sterility, or inviability. Nonetheless, molecular descriptions of such an intracellular conflict are rare, and its generality as an engine of speciation remains an open question.

Here, we present results of a systematic study aimed at understanding the role of cytonuclear incompatibility in postzygotic reproductive isolation. We screened three sensu stricto yeasts, S. cerevisiae, S. paradoxus, and S. bayanus for incompatible genes causing F2 hybrid sterility and used the information about how these genes diverged in function to reconstruct the evolutionary history of the species. We found that only a few strongly incompatible gene pairs have evolved between these species. Two of them, MRS1 and AIM22, were identified and the molecular mechanisms of incompatibility were characterized. By analyzing the mutations of the MRS1 gene leading to its functional divergence between two species at the nucleotide level, we show that only three mutations make major contributions. Finally, we show that the functional divergence of these incompatible genes is correlated with the phylogeny, suggesting that cytonuclear incompatibility not only represents a general mechanism of reproductive isolation but has also occurred repeatedly during yeast evolution.

Results
Cytonuclear Incompatibility between Three Saccharomyces sensu stricto Yeasts

A previous study has shown that incompatibility between a S. bayanus nuclear gene, AEP2, and S. cerevisiae mitochondria causes interspecific F2 hybrid sterility [14]. To examine whether nuclear-mitochondrial incompatibility represents a general mechanism of reproductive isolation in yeast, a systematic screen for such genes was conducted in three closely related yeast species: S. cerevisiae (Sc), S. paradoxus (Sp), and S. bayanus (Sb). Hybrid diploid strains between S. cerevisiae and S. paradoxus, or between S. cerevisiae and S. bayanus were induced to generate haploid spores containing different combinations of chromosomes from their parental species. These spores were then assayed for cytonuclear incompatibility. In hybrid diploids between species A and B, incompatibility could occur in two directions, between A-nucleus and B-mitochondria or between B-nucleus and A-mitochondria. When generating the yeast hybrids, we deliberately removed one parental type of mitochondria (by using rho0 mutants, which lack mitochondrial DNA) so that we could unambiguously assign the direction of incompatibility. After sporulation of hybrids, viable spores were measured for their ability to grow on glycerol, a non-fermentable carbon source (Figure 1). The results showed strong cytonuclear incompatibility in most of the interspecific hybrids; we observed 66±5%, 78±7%, and 32±16% of respiration-proficient spores in the interspecific crosses between Sc-rho0 and Sp, Sb-rh0 and Sc, and Sc-rho0 and Sb, respectively, while the intraspecific crosses generated almost 100% of respiration-proficient spores (Figure 2).

The percentage of viable spores that could grow on glycerol plates was used to estimate the number of strongly incompatible loci (see Materials and Methods). The results of this growth assay suggest that there are only one or few nuclear genetic loci strongly incompatible with mitochondria in the Sc-nucleus and Sp-mitochondria, the Sb-nucleus and Sc-mitochondria, and the Sb-nucleus and Sb-mitochondria pairs. Although no strong incompatibility was detected for the Sp-nucleus and Sc-mitochondria pair, slow spore growth (on glycerol plates) was commonly observed, suggesting that some incompatibility may exist in this pair as well. Because the incompatibility between the Sb-nucleus and Sc-mitochondria has been described earlier [14], we focus here on the remaining two pairs.

Identification of Two Cytonuclear Incompatible Genes Causing Reproductive Isolation

In our experimental design, we constructed the hybrid diploids using spo11Δ mutants to prevent meiotic homologous recombination. We expected that hybrid F1 haploids unable to respire (see Figure 1) should carry a specific set of chromosomes containing the incompatible genes. To identify the genes responsible for the observed cytonuclear incompatibility, the respiration-deficient clones were first examined for their chromosome contents using species-specific PCR (see Materials and Methods). In the cross
between Sc-\(\rho^0\) and Sb, the haploids carrying Sb-mitochondria but unable to respire lacked Sb-Chromosome 6, 9, or both (Table S1A). When these hybrid clones were transformed with genomic DNA libraries to screen for those genes capable of rescuing the respiratory defect, Sb-\(MRS1\) encoded on Sb-Chromosome 9 and Sb-\(AIB2\) encoded on Sb-Chromosome 6 were isolated. The results from the cross between Sc-\(\rho^0\) and Sp are more complex, as all the respiration-deficient clones did not contain the two Sp-Chromosomes 4 and 9 (Table S1B), but were fully rescued by Sp-\(MRS1\) alone, which is encoded on Sp-Chromosome 9. The reason why all the hybrid clones are missing Sp-Chromosome 4 is unclear. One possibility is that Sp-Chromosome 4 is also incompatible with Sc-Chromosome 9, an issue requiring further investigation.

Figure 1. A genetic screen for hybrid haploid clones carrying incompatible nuclear-mitochondrial genomes. One species (species A) was crossed to a \(\rho^0\) mutant of another species (species B) in which the mitochondrial DNA was completely deleted. In both strains, the \(SPO11\) genes were deleted to prevent meiotic recombination between homologous chromosomes. The hybrid diploids were then induced to sporulate and viable F1 spores were assayed for their ability to grow on glycerol plates (a non-fermentable carbon source). Respiration-deficient clones (Gly\(--\)) were then crossed to \(\rho^0\) mutants of the parental strains. If the Gly\(--\) phenotype is due to loss of mtDNA, the products from both crosses will be Gly\(\neg\). On the other hand, if the respiratory defect is caused by mutations occurring during the process, both products will be Gly\(\neg\). Only if the Gly\(--\) phenotype is caused by recessive incompatibility will crossing with the \(\rho^0\) mutant of species A rescue the defect (Gly\(+\)), while crossing with the \(\rho^0\) mutant of species B will not (Gly\(--\)). The Gly\(--\) clones containing incompatible cytonuclear genomes were further analyzed to determine their chromosomal contents (see Table S1). doi:10.1371/journal.pbio.1000432.g001
To rule out the possibility that the respiration defects caused by Sc-MRS1 and Sc-AIM22 were simply due to nuclear-nuclear incompatibility in the hybrid haploid clones, we crossed Sp-mrs1D, Sb-mrs1D, and Sb-aim22D with Sc-r0 and examined the respiratory ability of the diploid cells. All the diploid cells were still respiration-deficient, even though they contained a complete set of the S. cerevisiae genome (Figure 3). This result demonstrated that Sc-MRS1 is incompatible with Sp-mitochondria and that both Sc-MRS1 and Sc-AIM22 are incompatible with Sb-mitochondria.

Yeast cells utilize non-fermentable carbon sources to induce meiosis. Previous studies have shown that respiration-deficient cells were unable to sporulate [34]. To confirm that indeed the cytonuclear incompatibility observed in our experiments contributes to reproductive isolation, the aforementioned diploid cells (Sp-mrs1A×Sc-p0, Sb-mrs1A×Sc-p0, and Sb-aim22A×Sc-p0) were grown on sporulation medium and examined for their sporulation efficiency. No ascus was observed in these cultures, while the control cultures sporulated efficiently. Thus, cytonuclear incompatibility caused by Sc-MRS1 or Sc-AIM22 results in reproductive isolation between these yeast species.

**MRS1 Has Coevolved with the COX1 Introns**

Mrs1 is a mitochondrial protein required for excision of the a15β intron in COX1 and the b13 intron in COB [35,36]. A previous study has shown that S. douglasii Mrs1 (S. douglasii is a synonym of S. paradoxus) is required to splice a S. douglasii-specific COX1 intron not existing in the S. cerevisiae COX1 [37]. We observed a similar result in S. bayanus. Sc-MRS1 could not complement the respiratory defect of the Sp-mrs1A or Sb-mrs1A mutants (Figure S1). When Sb-Mrs1 or Sp-Mrs1 were replaced by Sc-Mrs1, the level of mature COX1 mRNA was drastically reduced but the COB mRNA was not affected (Figure 4A). We further analyzed the translation products from purified mitochondria and confirmed that only the Cox1 protein was missing (Figure 4B). By contrast, Sc-MRS1 transcription and protein transport into mitochondria appear to be normal in both S. paradoxus and S. bayanus (Figure 5). Thus the incompatibility between the mitochondrial and nuclear genomes is most likely due to a change in the splicing specificity of Mrs1 that occurred after S. cerevisiae diverged from the common ancestor of S. cerevisiae and S. paradoxus.

In order to understand how the COX1 introns evolved in yeast, we compared COX1 intron patterns between S. cerevisiae, S. paradoxus, S. bayanus, S. servazzii, Candida glabrata, and Kluyveromyces thermotolerans. S. servazzii and C. glabrata are species outside the sensu stricto complex and K. thermotolerans is a pre-WGD (whole-genome duplication) species. The comparison indicates that the intron in the Sp- or Sb-COX1 gene incompatible with Sc-Mrs1 is an ancient intron (Figure 4C). Since this intron was eliminated only in the S. cerevisiae lineage, it is likely that the Sc-MRS1 gene product lost the ability to splice this intron after the intron loss event (by adaptation or drift).

**The Functional Change of Mrs1 Is Mainly Caused by Three Nonsynonymous Mutations**

The fact that S. cerevisiae and S. paradoxus share a high degree of nucleotide sequence identity allowed us to determine the key mutations underlying the functional change of the MRS1 gene and
to reconstruct the process of Mrs1 evolution. To this end, chimeric proteins with regions from Sc-Mrs1 and Sp-Mrs1 were constructed and assayed for their ability to complement the respiratory defect of the Sp-mrs1Δ mutants. We found that the functional difference between Sc-Mrs1 and Sp-Mrs1 is mainly determined by a region comprising 63 amino acids (a.a. sites 179–241; Figure 6A). Nine

| Strain Combination          | YPD          | YEP+glycerol |
|----------------------------|--------------|--------------|
| Sb-mrs1Δ X Sc-r0           | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sb-mrs1Δ-p0 X Sc           | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sb X Sc-r0                 | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sb-p0 X Sc                 | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sp-mrs1Δ X Sc-r0           | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sp-mrs1Δ-p0 X Sc           | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sp X Sc-r0                 | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |
| Sp-p0 X Sc                 | ![YPD Image](image1) | ![YEP+glycerol Image](image2) |

**Figure 3.** The respiratory defects caused by Sc-MRS1 or Sc-AIM22 are not due to nuclear-nuclear incompatibilities. Hybrid diploid cells contain two complete sets of nuclear chromosomes but only one type of mitochondrial DNA: S. paradoxus mitochondrial DNA in Sp-mrs1Δ×Sc-r0 and Sp×Sc-r0, S. bayanus mitochondrial DNA in Sb-mrs1Δ×Sc-r0, Sb-aim22Δ×Sc-r0 and Sb×Sc-r0, and S. cerevisiae mitochondrial DNA in Sp-mrs1Δ-p0×Sc, Sp-p0×Sc, Sb-mrs1Δ-p0×Sc, Sb-aim22Δ-p0×Sc and Sb-p0×Sc. The hybrid cells were serially diluted and plated on YPD or glycerol plates to measure their growth. doi:10.1371/journal.pbio.1000432.g003
nonsynonymous changes have accumulated in this region since Sc-MRS1 and Sp-MRS1 diverged (Figure S2A). To determine which of these mutations led to the altered activity of Mrs1, we introduced the Sc. paradoxus version of each of these sites into Sc-MRS1 and assayed their ability to rescue the Sp-mrs1D respiratory defect. Analogous experiments were performed in the reverse direction by introducing Sc-specific amino acids into Sp-MRS1. Only mutations in three amino acids (Sc to Sp: T201A, V211A, and M227I) had obvious contributions (Figure 6B and Figure S2). When all three mutations were combined together in a single mutant clone, it explained most of the effect. The growth rate of cells carrying the mutant plasmid (Sc-MRS1-n123) in a glycerol-containing medium is about 75% of that of wild type cells. Interestingly, these three amino acids are all conserved in Sc-AIM22, S. kudriavzevii, and Sc-AIM22, but are changed in Sc. cerevisiae. Among the other six nonsynonymous changes in this region, only two of them (Sc to Sp: K186E and R223Q) share the same pattern. This observation is consistent with our hypothesis that the functional change of MRS1 occurred only after an ancestral COX1 intron was lost in Sc. cerevisiae. Our results clearly demonstrate that the observed nuclear-mitochondrial incompatibility results from cumulative effects of multiple mutations. On the other hand, they also suggest that only a small fraction of the nonsynonymous changes between species contributes to the incompatibility.

The Mrs1 protein does not contain any specific functional domain. However, a recent study has used computer modeling to predict the Mrs1 protein structure [38]. We examined the relative positions of these residues using the predicted structure. All three residues (a.a 201, 211, and 227) were found to localize on the RNA-binding surface (Figure S3). It is possible that these amino acid changes have altered the substrate specificity of Mrs1 that leads to the incompatibility.

Sc-AIM22 Is Not Compatible with Sb-Mitochondria

S. cerevisiae nuclei can only support S. bayanus mitochondria if the cells contain a S. bayanus AIM22 gene. AIM22 encodes a lipoate-protein ligase homologous to the bacterial lplA protein [39,40]. In eukaryotic cells, lipoic acid has been shown to be an essential cofactor to a variety of mitochondrial proteins and lipoate-protein ligase
(together with other enzymes) is required to lipoylate these mitochondrial targets [39,41]. We have not investigated which mitochondrial protein or enzyme in Sb-mitochondria is incompatible with Sc-Aim22. However, our data indicate that the incompatibility is not caused by misregulation of the Sc-AIM22 transcription or failure to transport the Sc-Aim22 to Sb-mitochondria (Figure 5).

To investigate whether the AIM22 gene in the S. cerevisiae–S. paradoxus branch has been under positive selection during evolution, we measured the ratio of nonsynonymous (Ka) to synonymous (Ks) nucleotide substitution rates between these species. The Ka/Ks values of AIM22 in the S. cerevisiae–S. paradoxus, S. cerevisiae–S. bayanus, and S. paradoxus–S. bayanus pairs...
are 0.13, 0.12, and 0.14, showing no sign of positive selection (Ka/Ks > 1). We also ran a PAML’s branch-model analysis on the AIM22 gene [42,43] but could not detect any signature of significant positive selection (Figure S4).

Previous studies of AEP2 have shown that the nuclear-mitochondrial incompatibility is asymmetrical. While Sb-Aep2 is completely incompatible with Sc-mitochondria, Sc-Aep2 retains partial compatibility with Sb-mitochondria [14]. We also found that incompatibility caused by AIM22 and MRS1 only occurred in one direction. Although Sc-AIM22 and Sc-MRS1 are not compatible with Sb-mitochondria, Sb-AIM22 could complement the Sc-am22A mutant and both Sb- and Sc-MRS1 could rescue the Sc-mrs1A mutant.

The Evolution of Cytonuclear Incompatibility Is Correlated with the Phylogeny

Nuclear-mitochondrial incompatibility has been shown to occur commonly between different yeast species [32,33]. However, it is unclear whether nuclear-mitochondrial incompatibility between different pairs of species has evolved at different periods of time in different species lineages. To address this issue, we tested the compatibility between different orthologues of these incompatible genes and mitochondria from each species. Different orthologous alleles of MRS1, AIM22, or AEP2 were transformed into the S. cerevisiae, S. paradoxus, or S. bayanus mutants in which the wild-type copy had been deleted. The transformants were then tested for their ability to grow on glycerol plates (Figure 7A and Figure S1). Information from these assays was used to deduce the time of occurrence of the functional change leading to incompatibility. A clear correlation between the emergence of cytonuclear incompatibility and the phylogeny is observed (Figure 7B). Sc-MRS1 is incompatible with Sp-mitochondria or Sb-mitochondria, indicating that the functional change of Sc-MRS1 occurred only in the S. cerevisiae lineage. On the other hand, the functional change of AIM22 represents a more ancient event in the common ancestor of S. cerevisiae and S. paradoxus, because Sc-AIM22 and Sb-AIM22 are exchangeable but neither of them is compatible with Sb-mitochondria. Finally, the data suggest that Sb-AEP2 diverged in function only in the S. bayanus lineage since Sb-AEP2 is incompatible with either Sc-mitochondria or Sp-mitochondria. These results provide evidence that nuclear-mitochondrial incompatibility has repeatedly arisen during the history of yeast evolution and probably represents an important reproductive isolation mechanism in yeast species.

Discussion

Previous studies in Saccharomyces yeasts have suggested that the deleterious effect of DNA sequence divergence on meiotic recombination probably contributes in part to reproductive isolation during yeast evolution [12,44,45]. Our results, on the other hand, suggest that nuclear-mitochondrial incompatibility is also a promising candidate for causing intrinsic hybrid dysfunction. In fact, these two mechanisms are not mutually exclusive. Genomes from different populations will accumulate enough DNA sequence divergence only after extended periods of allopatric isolation, but the effect of sequence divergence can be applied directly in the diploid F1 hybrid cells. Cytonuclear incompatibility can be achieved by only a few mutations, and its deleterious effect can be carried on to the F1 gamete or F2 progeny. In theory, cytonuclear incompatibility has a stronger impact on blocking gene flow between populations in the early stages of speciation,
and reproductive isolation is reinforced later on when populations have accumulated enough DNA sequence divergence. These two mechanisms can be complementary to each other in terms of their effects and evolutionary trajectories. It will be interesting to investigate whether cytonuclear incompatibility exists between different populations of the same species.

Reproductive isolation resulting from genetic incompatibility has been discovered in a variety of organisms [7]. Most of the examples characterized so far are caused by interactions between nuclear genes. In yeast, this type of incompatibility has been investigated in a few studies, yet no strongly nuclear-nuclear incompatible genes were identified [14,46]. On the other hand, cytonuclear incompatibilities were observed in hybrids between different yeast species or populations [47]. Cytonuclear incompatibility probably represents a more general mechanism of reproductive isolation in yeast. By analyzing the functions and the interacting components of the identified incompatible genes, we discovered that cytonuclear incompatibility could be achieved by multiple molecular mechanisms: intron splicing, protein lipoylation, and activation of mRNA translation. This suggests that cytonuclear incompatibility in yeast can occur in various pathways by diverse molecular mechanisms.

![Figure 7](https://example.com/figure7.png)

**Figure 7. The evolution of incompatibility is correlated with the phylogeny.** (A) Results of the functional complementation assay indicate that the functional change of Mrs1, Aim22, and Aep2 occurred at different periods of time. Different orthologous alleles of MRS1, AIM22, or AEP2 were transformed into S. cerevisiae, S. paradoxus, or S. bayanus mutants in which the wild-type copy had been deleted. The transformants were then tested for their ability to grow on glycerol plates. If the transformant was able to grow on the glycerol plate, it suggested that the mitochondria were compatible with the gene (which is indicated by the plus sign). Sc-mito, Sc-mitochondria; Sp-mito, Sp-mitochondria; Sb-mito, Sb-mitochondria. (B) A diagram of the phylogeny of three closely related Saccharomyces species showing the branches in which functional changes of the cytonuclear incompatible genes have occurred and the molecular functions of these genes.

doi:10.1371/journal.pbio.1000432.g007
Scientists usually compare orthologous sequences from different species and use the detected molecular signatures to infer the evolutionary process of a gene. Since our data suggest that Mrs1 changed function only after S. cerevisiae diverged from S. paradoxus, it is reasonable to assume that the altered function originated from amino acid changes that occurred specifically in the S. cerevisiae lineage. By comparing the coding regions of the MRSL orthologues from S. cerevisiae, S. paradoxus, S. kudriavzevia, and S. bayanus, we observed 22 amino acids that are different in S. cerevisiae but are conserved in the other three species. Interestingly, our functional assays showed that only three of these amino acid changes contribute significantly to the functional differences of Mrs1. The other mutations may have very minor effects unable to be detected by our functional assays or have been fixed simply by genetic drift. In a previous study, Rawson and Burton have also observed that three amino acid changes in a nucleus-encoded cytochrome c (CYC) are responsible for cytonuclear incompatibility between different populations of a marine copepod, Tigriopus californicus [48]. These results illustrate the importance of mapping the critical amino acid changes in order to understand how a gene evolved.

What is the major driving force underlying the evolution of mitochondria? It is known that the mitochondrial genome suffers a higher mutation load because it is constantly facing higher levels of oxidative reagents and its DNA protection system is more primitive as compared to the nuclear genome. With a much smaller copy number of mitochondrial DNA in yeast (30–80 molecules of mitochondrial genomes in yeast cells compared to 1,000–5,000 molecules in animal cells), mutations may be fixed frequently in the mitochondrial genome by genetic drift. Since wild yeast often propagate clonally in natural environments [13], a founder cell with mild deleterious mutations in its mitochondrial genome may have a chance to accumulate suppressors in the mitochondrial or nuclear genomes to rescue the fitness before its progeny are outcompeted by cells from another population. Alternatively, mitochondrial evolution may be driven by an “arms race” process between selfish mitochondrial DNA and the “wild-type” mitochondrial or nuclear genomes. It is commonly observed in yeast that by manipulating the host replication or segregation systems, some mitochondrial genomes allow themselves to be inherited more efficiently, even though they may be carrying compromised respiratory functions [49]. In a sexual population, the other mitochondrial or host nuclear genomes will be selected to counteract this selfish behavior or the deleterious effects carried by the selfish DNA. Such an “arms race” may allow mitochondrial genomes to evolve faster than by genetic drift (in a fashion similar to positive selection). Incompatibilities driven by arms races between different genetic components have been suggested in a few recent studies [7]. Among these genetic conflicts, most of them are caused by selfish elements manipulating segregation distortion [11,50,51,52,53]. The “hybrid necrosis” phenotype observed in Arabidopsis probably results from recurrent conflicts between the host defense system and pathogens [10]. The genetic conflict caused by selfish mitochondrial genomes may represent another type of arms race. It will be interesting to investigate whether the arms race model can explain the cytonuclear incompatibilities observed in other organisms. Finally, ecological adaptation may also contribute to mitochondrial evolution. Evidence suggests that adaptive mutations have occurred in mitochondria in response to different environmental stresses that interfere with cellular energy demands [48,34,55]. In yeast, it has been shown that S. bayanus grows much better than S. cerevisiae on media containing only non-fermentable carbon sources, with the opposite observed in fermentable media. It has been speculated that the changes in Sb-AEP2 and Sb-OL11 are a part of such ecological adaptation [14].

Reciprocal crosses between species often generate asymmetrical hybrid viability or sterility, a general feature of intrinsic postzygotic isolation called Darwin’s corollary [56,57,58,59,60]. The Dobzhansky-Muller model suggests that alleles causing reproductive isolation act asymmetrically [61]. However, asymmetries in allele action do not necessarily lead to asymmetries in reproductive isolation [62]. Incompatibility between autosomal loci affects both reciprocal crosses identically. Asymmetric reproductive isolation is usually caused by incompatibility between autosomal loci and unparentally inherited materials, such as cytoplasmic elements and sex chromosomes. It has been suggested that cytonuclear incompatibility caused by different trajectories of mitochondrial evolution in different species may contribute to this phenomenon [59,63]. Our results provide the molecular basis to support this hypothesis. Since cytonuclear incompatibility can be achieved by multiple molecular mechanisms and evolve at different rates in different lineages, it can serve as a general mechanism of reproductive isolation and also create asymmetrical reproductive isolation between species.

Materials and Methods

Strains and Genetic Procedures

Yeast strain genotypes are listed in Table S2. The parental S. cerevisiae strains [YJLI127 and YJLI128] are isogenic with W303 (MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100). The parental S. paradoxus strains [YJLI119 and YJLI138] are derived from YD9 and are a gift from Dr. Duncan Greig (University College London, UK). The parental S. bayanus strains [YJLI1130 and JYLI1031] were derived from a strain (S. bayanus #180) collected by Dr. Duccio Cavaliere (University of Florence, Italy). The strains JYLI1157, 917, and 1256 were used for measuring hybrid fertility. Substitutive and integrative transformations were carried out by the lithium acetate procedure [64]. Media, microbial, and genetic techniques were as described [65].

Estimation of the Nuclear-Mitochondrial Incompatible Gene Number between Different Species

a and α cells of one species (S. cerevisiae, S. paradoxus, or S. bayanus) were crossed to α and a p cells of another species to generate F1 hybrid cells. In both species, the SPO11 genes were deleted to prevent meiotic recombination between homologous chromosomes. Strains from the first species also had a URA3 marker inserted near the centromere of a chromosome so that haploid spores could be efficiently selected on 5-FOA plates. After the hybrid diploids were induced to sporulate, viable spores were tested for their respiratory ability. Respiration-deficient clones were further crossed with p0 mutants of the parental species to confirm that the defect was caused by cytonuclear genomic incompatibility (Figure 1). A genetic analysis was used to estimate the number of the nuclear genes that are incompatible with mitochondrial DNA from another species: in a cross between two spo11A mutants, meiotic recombination does not occur and homologous chromosomes segregate randomly [66,67]. For a specific chromosome A, 25% of the spores will carry two A chromosomes, 50% of them will carry one A chromosome, and 25% of them will have no A chromosome (these cells will not survive so they will not be counted in our later analysis). If only one gene on chromosome A is recessively incompatible with mitochondrial DNA (in our case, all spores from a single cross carry the same parental type of mitochondrial DNA), we expect to see that 66% of the viable spores are Gly+ (1/3×2/3×1/2 = 66%). The spores carrying two A chromosomes should be Gly+ because the incompatibility is recessive. Half of the spores
that carry only one A chromosome should be Gly+ if their A chromosome is from the same parent of the mitochondrial DNA. If two genes on different chromosomes are involved, we expect to see that 43% of the viable spores are Gly+ (66% × 66% = 43%). Epistatic effects are not taken into account in this analysis because considering such effects would make it impossible to estimate the involved locus number.

Genetic Screens and Genotyping of the Hybrid Haploids with Incompatible Nuclear-Mitochondrial Genomes

We examined the chromosome composition of respiration-deficient hybrid lines by PCR using species-specific primers for all 16 chromosomes. Yeast genomic DNA libraries constructed from S. bayanus or S. paradoxus genomes were transformed into the Gly− clones to screen for incompatible genes.

RNA Isolation and Northern Analyses of Mitochondrial Transcripts

Yeast strains were grown in 3 ml YPD liquid cultures at 30°C to stationary phase and total RNA was isolated using Qiagen RNeasy Midi Kits (Qiagen, Valencia, CA). Ten μg of total RNA was separated on a 1.3% agarose-formaldehyde gel and then visualized by using Western Lightning chemiluminescence reagent (PerkinElmer, Waltham, MA). Anti-G6PDH polyclonal antibody was purchased from Sigma (St. Louis, MO). Anti-c-Myc polyclonal antibody (A-14) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Hsc82 and Anti-Atp2 polyclonal antibodies were obtained from Dr. Chung Wang (Academia Sinica, Taipei).

In Vivo Radiolabeling of Mitochondrial Proteins

Cells in the early log phase were inoculated in 2 ml standard minimal medium and grown for 30 min. Cycloheximide stock solution (10 μl/ml in dH2O) was added to a final concentration of 100 μg/ml. Cells were incubated for 5 min prior to addition of 0.1 mM of [35S]-methionine. The reaction was terminated after 1 h by adding 2 ml of chase solution (1% casamino acid, 2 mg/ml Na2SO4). Mitochondria were prepared as described [65]. The radiolabeled proteins were separated on a 17.5% polyacrylamide gel.

Prediction of the Ancestral AIM22 Sequence

The ancestral AIM22 sequence of S. cerevisiae and S. paradoxus was constructed using a maximum likelihood procedure (free-ratio model) as implemented in the PAML package. The Ka/Ks ratios were calculated using DNAsp 5.0 [71].

Branch-Model Analysis for AIM22

The Ka/Ks ratios were estimated using the free-ratio branch model of PAML. This method allows the Ka/Ks ratios to vary among branches in a given phylogeny and is useful in detecting positive selection acting on particular lineages [42]. The AIM22 sequences from five Saccharomyces sensu stricto species (S. cerevisiae, S. paradoxus, S. mikatae, S. bayanus, and S. kudriavzevi) were used in our analysis. Results of the branch-model PAML analysis suggested that no particular lineage was subjected to positive selection (i.e., Ka/Ks>1) (Figure S4). Because both Sc-AIM22 and Sp-AIM22 are incompatible to S. bayanus mitochondria, it is possible that there has been an accelerated sequence evolution in either the lineage leading to S. cerevisiae and S. paradoxus, or in the lineage leading to S. bayanus. To test this hypothesis, we performed an analysis as described in Yang and Nielsen [43]. One ω-ratio and two ω-ratio branch models were implemented. The first model assumes that only one ω-ratio leads to whole phylogeny branches and the second model assumes that one ω-ratio leads to the branch that we are interested in and another ω-ratio leads to the rest of the other branches. Twice the difference of their likelihood ratio between any two models (likelihood ratio test; LRT) was then compared against a chi-square distribution. The degree of freedom (d.f.) was obtained based on the difference of parameters used in two different models. From our analysis, no accelerated evolution was observed in any lineage along the phylogeny of AIM22.

Supporting Information

Figure S1  Functional complementation assays of different orthologous MRS1 and AIM22 genes. Orthologous MRS1 and AIM22 genes from different species were cloned into single-copy plasmids and then transformed into the S. cerevisiae, S. paradoxus, or S. bayanus mutants in which the wild-type copy had been deleted. The transformants were serially diluted and plated on YPD or glycerol plates to measure their growth. The empty vector was used as a control in the experiment.

Figure S2  Growth rates of the S. paradoxus mrs1A mutants carrying different hybrid or mutant MRS1
genes in glycerol-containing media. (A) Protein sequence alignment between Sp-Mrs1 and Sc-Mrs1. Only the region between amino acids 177 and 241 is shown. The numbers indicate positions of the nonsynonymous changes. (B) Growth rates of the S. paradoxus mrs1A mutants carrying different mutant Sc-MRS1 genes in glycerol-containing media. Each of Sc-MRS1 mutants contains a single amino acid change (from Sc to Sp). (C) Growth rates of the S. paradoxus mrs1D mutants carrying different mutant H-MRS1 genes in glycerol-containing media. Each of H-MRS1 mutants contains a single amino acid change (from Sp to Sc). Cells were pre-adapted to the glycerol-containing medium for 1 d and those in the early log phase were used to measure the growth rate. Three independent replicates for each fitness measurement were performed.

Figure S3 The critical nonsynonymous changes occur at the residues positioned on the RNA-binding surface. A structural model for the Mrsl protein-RNA complex. The RNA backbone is shown as a tube and the domains protected by Mrsl is shown as red spheres on the Mrsl proteins. The three critical amino acids with nonsynonymous substitutions (201, 211, and 227) are shown as red spheres on the Mrs1 proteins.

Figure S4 Evolutionary rate analysis of AIM22 from different Saccharomyces yeast species. (A) The Ka/Ks ratios for the entire AIM22 coding sequence in different Saccharomyces lineages. The tree branch length and branch-wise Ka/Ks ratio were estimated using a free-ratio model by Codeml in the PAML package. (B) The lineage that leads to S. cerevisiae and S. paradoxus was treated as foreground branches (02) and the other as background branches (01). When 02 was compared with 01, no significantly accelerated rate was detected using LRT (p = 0.5902). (C). The lineage that leads to S. bayanus was treated as foreground branches (02) and the other as background branches (01). No significantly accelerated rate was detected between these two branches (p = 0.1407). 2B, twice the difference of likelihood values from two models; dF, degree of freedom; H0, null model; H1, alternative model.

Table S1 Chromosome genotyping of the hybrid haploids with deficient mitochondrial functions. Genomic DNAs isolated from the F1 Gly− haploid cells (Figure 1) were analyzed by PCR with species-specific primers for each chromosome (+, chromosome detected). After MRS1 and AIM22 were identified to cause the cytonuclear incompatibility, plasmids carrying Sb-MRS1, Sb-AIM22, Sb-MRS1-AIM22, or Sp-MRS1 were transformed into each clone to see whether the respiratory defect could be rescued (+, rescued; ×, not rescued; nd, not determined).

Table S2 Yeast strains.

Acknowledgments

We thank E. Louis, A. Murray, and members of the Leu lab for helpful discussions and comments on the manuscript. We thank H. Kuhn and H. Wilson for English editing. We thank S.-L. Liu for advice on PAML analyses, and C. Duncan and K. Weeks for providing the information on Mrsl protein structure.

Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: JYL. Performed the experiments: JYC, YSH KHI, HYL, JYL. Analyzed the data: JYC YSH KHL HYL, JYL. Contributed reagents/materials/analysis tools: JYL. Wrote the paper: JYC JYL.

References

1. Coyne JA, Orr HA (2004) Speciation. Sunderland, MA: Sinauer Associates.
2. Dobzhansky T (1937) Genetics and the origin of species. New York: Columbia University Press.
3. Muller H (1942) Isolating mechanisms, evolution, and temperature. Biological Symposia 6: 71–125.
4. Noor MA, Feder JL (2006) Speciation genetics: evolving approaches. Nat Rev Genet 7: 851–861.
5. Wu CI, Ting CT (2004) Genes and speciation. Nat Rev Genet 5: 114–122.
6. Mallet J (2006) What does Drosophila genetics tell us about speciation? Trends Ecol Evol 21: 386–393.
7. Persgraves DC (2010) The molecular evolutionary basis of species formation. Nat Rev Genet 11: 175–180.
8. Bikard D, Patel D, Le Mette C, Giorgi V, Camilleri C, et al. (2009) Divergent evolution of duplicate genes leads to genetic incompatibilities within A. thaliana. Science 323: 623–626.
9. Schard M (2008) Evolution of Xmrk: an oncogene, but also a speciation gene? Bioscien 30: 822–822.
10. Bomblies K, Weigel D (2007) Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. Nat Rev Genet 8: 382–392.
11. Mihola O, Trachtulec Z, Vlkov C, Schimenti JC, Forejt J (2009) A mouse speciation gene encodes a mitoctic histone H3 methyltransferase. Science 325: 373–375.
12. Greig D (2009) Reproductive isolation in Saccharomyces. Heredity 102: 39–44.
13. Greig D, Lee JY (2009) Natural history of budding yeast. Curr Biol 19: R906–R909.
14. Lee HY, Chou JY, Cheong L, Chang NH, Yang SY, et al. (2008) Incompatibility of nuclear and mitochondrial genomes causes hybrid sterility between two yeast species. Cell 135: 1065–1073.
15. Yamagata Y, Yamamoto E, Aya K, Win KT, Doi K, et al. (2010) Mitochondrial gene in the nuclear genome induces reproductive barrier in rice. Proc Natl Acad Sci U S A 107: 1494–1499.
16. Fishman L, Wills JH (2006) A cytonuclear incompatibility causes another sterility in Mimulus hybrids. Evolution 60: 1372–1381.
17. Hanso MR, Bentolila S (2006) Interactions of mitochondrial and nuclear genes that affect male gametophyte development. Plant Cell 16 Suppl: S154–S169.
18. Anderson SG, Zomorodipour A, Anderson JO, Sicheritz-Ponten T, Almquist UC, et al. (1998) The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396: 133–140.
19. Martin W, Herrmann RG (1998) Gene transfer from organelles to the nucleus: how much, what happens, and why? Plant Physiol 118: 9–17.
20. Dieckmann CL, Staples RR (1994) Regulation of mitochondrial gene expression in Saccharomyces cerevisiae. Int Rev Cytol 152: 145–181.
21. Grivell LA (1989) Nucleo-mitochondrial interactions in yeast mitochondrial biogenesis. Eur J Biochem 182: 477–493.
22. Reinders J, Zahedi RF, Plunmer N, Meisinger C, Sickmann A (2006) Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. J Proteome Res 5: 1543–1554.
23. Rand DM, Haney RA, Fry AJ (2004) Cytoplasmic correlation: the genomics of cooperation. Trends Ecol Evol 19: 645–653.
24. Blier PC, Dufresne F, Burton RS (2004) Natural selection and the evolution of miRNA-encoded peptides: evidence for intergenomic co-adaptation. Trends Genet 17: 400–406.
25. Gershoni M, Templeton AR, Mishmar D (2009) Mitochondrial bioenergetics as a major motive force of speciation. Bioscience 31: 642–650.
26. Barrientos A, Kenyon L, Moraes CT (1998) Human xenon mitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. J Biol Chem 273: 14210–14217.
27. Chase CD (2007) Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends Genet 23: 81–90.
28. Ellison CK, Burton RS (2006) Disruption of mitochondrial function in interpopulation hybrids of Tigriopus californicus. Evolution Int J Org Evolution 60: 1302–1391.
29. Ellison CK, Nicholls O, Gadad J (2008) Hybrid breakdown and mitochondrial dysfunction in hybrids of Nasiona parasitoid wasps. J Ecol Biol 21: 1844–1851.
30. Lépinas A, Hennen S (1977) Cytochrome oxidase deficiency during development of amphibian nucleocytoplasmic hybrids. Dev Biol 57: 284–292.
31. Sackton TB, Haney RA, Rand DM (2003) Cytonuclear coadaptation in Drosophila: disruption of cytochrome c oxidase activity in backcross genotypes. Evolution Int J Org Evolution 57: 2315–2325.
32. Spirek M, Horvath A, Piskur J, Sulo P (2000) Functional co-operation between the nuclei of Saccharomyces cerevisiae and mitochondria from other yeast species. Curr Genet 38: 202–207.
33. Sulo P, Spirek M, Solteszova A, Marinioni G, Piskur J (2003) The efficiency of functional mitochondrial replacement in Saccharomyces species has directional character. FEMS Yeast Res 4: 97–104.
34. Kureza MT, Tingle MA, Halverson HO (1974) Sporulation of Saccharomyces cerevisiae in the absence of a functional mitochondrial genome. J Bacteriol 117: 89–98.
35. Kreile, J. Schulme, M. Pillar T, Korte A, Rodel G (1996) Cloning of a nuclear gene MRS1 involved in the excision of a single group I intron (b13) from the mitochondrial COB transcript in S. cerevisiae. Curr Genet 11: 185–191.
36. Bounouet I, Dochard G, Poyton RO, Sloininisi PP (1996) Two group I mitochondrial introns in the cob-box and cod genes require the same MRS1/PET157 nuclear gene product for splicing. Curr Genet 18: 117–124.
37. Herbert CJ, Macadre C, Becam AM, Lazowska J, Sloininisi PP (1992) The MRS1 gene of S. douglasii: co-evolution of mitochondrial introns and specific splicing proteins encoded by nuclear genes. Gene Expr 2: 203–214.
38. Duncan CD, Weeks KM (2010) The Msrl splicing factor binds the b1 group I intron at each of two tetraloop-receptor motifs. PLoS One 5: e9683. doi:10.1371/journal.pone.0009803.
39. Schonauer MS, Kastanottis AJ, Kursu VA, Hiltunen JK, Dieckmann CL (2009) Lipoyl acid synthesis and attachment in yeast mitochondria. J Biol Chem 284: 23234–23242.
40. Miller JR, Busby RW, Jordan SW, Cheek J, Henshaw TF, et al. (2000) Escherichia coli LipA is a lipoyl synthase: in vitro biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. Biochemistry 39: 15166–15178.
41. Marvin ME, Williams PH, Cashmore AM (2001) The isolation and characterisation of a Saccharomyces cerevisiae gene (LIP2) involved in the attachment of lipoyl acid groups to mitochondrial enzymes. FEMS Microbiol Lett 199: 131–136.
42. Yang Z, Swanson WJ, Vazquez GD (2000) Maximum-likelihood analysis of molecular adaptation in alabamid sperm lymin reveals variable selective pressures among lineages and sites. Mol Biol Evol 17: 1446–1453.
43. Yang Z, Nielsen R (2002) Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol Biol Evol 19: 908–917.
44. Hunter N, Chambers SR, Louis EJ, Borts RH (1996) The mismatch repair system contributes to mitotic sterility in an interspecific yeast hybrid. Embry J 15: 1726–1733.
45. Liti G, Burton DB, Louis EJ (2006) Sequence diversity, reproductive isolation, and species concepts in Saccharomyces. Genetics 174: 839–850.
46. Greig D (2007) A screen for recessive splicing genes expressed in the gametes of F1 hybrid yeast. PLoS Genet 3: e21. doi:10.1371/journal.pgen.0030021.
47. Chou JY, Lee JY (2010) Speciation through cytonuclear incompatibility: insights from yeast, and implications for higher eukaryotes. Bioessays 32: 401–411.
48. Rawson PD, Burton RS (2002) Functional coadaptation between cytochrome c and cytochrome c oxidase in allopatric populations of a marine copepod. Proc Natl Acad Sci U S A 99: 12953–12958.
49. Contamine V, Picard M (2000) Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol Mol Biol Rev 64: 281–315.
50. Ferrera PM, Barba PA (2009) Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in Drosophila. PLoS Biol 7: e1000234. doi:10.1371/journal.pbio.1000234.
51. Phadnis N, Orr HA (2009) A single gene causes both male sterility and segregation distortion in Drosophila hybrids. Science 323: 376–379.
52. Bayes JJ, Malick HS (2009) Altered heterochromatin binding by a hybrid sterility protein in drosophila sibling species. Science 326: 1538–1541.
53. Tang S, Presgraves DC (2009) Evolution of the Drosophila nuclear pore complex results in multiple hybrid incompatibilities. Science 323: 779–782.
54. Dowling DK, Friburg U, Lindell J (2009) Evolutionary implications of non-neutral mitochondrial genetic variation. Trends Ecol Evol 25: 546–554.
55. Ryan MT, Hoogenraad NJ (2007) Mitochondrial-nuclear communications. Annu Rev Biochem 76: 701–722.
56. Bolnick DI, Near TJ (2004) Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). Evolution 59: 1754–1767.
57. Darwin C (1859) On the origin of species. London: John Murray.
58. Tiffin P, Olson MS, Moyle LC (2001) Asymmetrical crossing barriers in angiosperms. Proc Biol Sci 268: 861–867.
59. Turelli M, Moyle LC (2007) Asymmetric postmatting isolation: Darwin’s corollary to Haldane’s rule. Genetics 176: 1039–1088.
60. Wu CI, Davis AW (1993) Evolution of postmating reproductive isolation: the composite nature of Haldane’s rule and its genetic bases. The American Naturalist 142: 187–212.
61. Coyne JA, Orr HA (1998) The evolutionary genetics of speciation. Philos Trans R Soc Lond B Biol Sci 353: 287–305.
62. Levin DA (1998) The origin of isolating mechanisms in flowering plants. Evol Biol 11: 185–315.
63. Bolnick DI, Turelli M, Lopez-Fernandez H, Wainwright PC, Near TJ (2008) Accelerated mitochondrial evolution and "Darwin’s corollary": asymmetric viability of reciprocal F1 hybrids in Cenarchid fishes. Genetics 178: 1037–1088.
64. Ito H, Fukada Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153: 163–168.
65. Guthrie C, Fink G (2004) Guide to yeast genetics and molecular and cell biology. San Diego: Elsevier Academic Press.
66. Klein F, Mahr P, Galova M, Buonomo SB, Michaelis C, et al. (1999) A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell 98: 91–103.
67. Shown MA, McCarroll R, Murray AW (2000) Requirement of the spindle checkpoint for proper chromosome segregation in budding yeast meiosis. Science 289: 300–303.
68. Sambrook J, Russell DW (2001) Molecular cloning. New York: Cold Spring Harbor Laboratory Press.
69. Rodsheller MS, Boone BE, Bryan AE, Shadel GS (2001) Nmplp1, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. J Biol Chem 276: 8616–8622.
70. Lemaire C, Dochard G (2008) Preparation of respiratory chain complexes from Saccharomyces cerevisiae wild-type and mutant mitochondrial activity measurement and subunit composition analysis. Methods Mol Biol 432: 65–81.
71. Liberato P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.