Post-zygotic sterility and cytonuclear compatibility limits in S. cerevisiae xenomitochondrial cybrids

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INTRODUCTION

Understanding the genetic basis of speciation is one of the major tasks in evolutionary biology. In spite of the recent methodological progress, many questions still remain unanswered or debated (Nosil and Schluter, 2011; Butlin et al., 2012). In general, several types of reproductive isolation have been considered, but most genetic studies are focused on the speciation process derived from interspecific hybrids. Consequently, the cause of the inviability and sterility resulting from the post-zygotic barrier has been studied (Dobzhansky, 1937; for a review see Butlin et al., 2012). Yeasts, particularly Saccharomyces cerevisiae, are the experimental model of choice for such speciation studies due to their ability to mate with related species and their homothallism, allowing the mating type switch in haploid cells after almost every division. Therefore, interspecies hybrids between different Saccharomyces species are able to establish fertile lines of “new species” after repeated cycles of sporulation and self-fertilization (Greig et al., 2002; reviewed in Greig, 2009; Chou and Leu, 2010; Louis, 2011; Morales and Dujon, 2012). In Saccharomyces yeasts, the reproductive barrier is post-zygotic and this can be attributed to non-collinear chromosomes (Delneri et al., 2003; Charon et al., 2014; Hou et al., 2014) “anti-recombination” resulting from the action of a “confused” mismatch repair system (Hunter et al., 1996; for a review see Greig, 2009; Louis, 2011).

In general, the divergence of species results from responses to negative epistatic genetic interactions, known as “Dobzhansky–Muller incompatibilities,” which are alleles that are completely normal in their native genetic background but are deleterious on the genetic background of the other population. The hypothesis was confirmed experimentally and reduced efficiency of meiotic reproduction was observed in hybrids made from populations adapted to two divergent environments (Dettman et al., 2007) and later some genes responsible for the divergence were identified (Anderson et al., 2010). So far, no Dobzhansky–Muller nuclear incompatibilities have been found in yeasts (Greig, 2007; Kao et al., 2010), but they have been reported between mitochondrial and nuclear genes (reviewed in Lee et al., 2008; Chou and Leu, 2010; Chou et al., 2010).
Functional mitochondria are indispensable for yeast sporulation, which is the essential step in hybrid speciation. The fate of mitochondrial DNA (mtDNA) has been largely overlooked in hybrid speciation lines, but it is believed that progeny inherit a complete mitochondrial (mt) genome from one of the yeast species, as has been described in natural or in vitro created hybrids (Marinoni et al., 1999; Rainieri et al., 2008; Solieri et al., 2008). Mitochondria have a limited genome, and efficient oxidative phosphorylation requires co-adaptation of mitochondrial and nuclear genes, despite divergent paces of evolution. Their biogenesis depends on the tender interplay of up to thousand proteins encoded by the nucleus, with eight proteins and several dozen gene products encoded by Saccharomyces mtDNA (for a review see Lipinski et al., 2010; Schmidt et al., 2010; Fox, 2012).

Consequently, in interspecific hybrids the mitochondrial genome from one partner does not have to communicate equally well with the nuclear genome of the second partner and the progeny can be sterile or non-viable. Such nucleo-mitochondrial communication has been reported as a determinant of reproductive isolation during yeast evolution (Lee et al., 2008; Chou et al., 2010; reviewed in Chou and Leu, 2010; Solieri, 2010). Pairs of genes with interspecific incompatibility were found using non-respiring chimeras containing mitochondria from one partner as well as a set of original chromosomes, where one or two were replaced with their counterpart from second partner (Lee et al., 2008; Chou et al., 2010). Then, the elements of interspecific nuclear-mitochondrial incompatibility can be recovered from genomic DNA libraries according to the ability to rescue the respiratory defect. This approach has revealed several Dobzhansky–Muller pairs: (i) Saccharomyces bayanus nuclear AEP2 allele with S. cerevisiae/Saccharomyces paradoxus mitochondrial atp9 gene; (ii) S. cerevisiae MRS1 nuclear gene required for the splicing of some mitochondrial group I introns with S. paradoxus; (iii) S. cerevisiae/S. paradoxus AIM22 gene variants coding enzyme required for the addition of lipoate to some mitochondrial proteins imported from cytoplasm with S. bayanus mitochondria (Chou et al., 2010). In spite of providing valuable data, this approach failed in the preparation of an entire set of chimeras with single chromosome replacement implicating additional Dobzhansky–Muller pairs (Lee et al., 2008; Chou et al., 2010).

Dobzhansky–Muller cytonuclear incompatibilities can generally be directly studied by the transplacement of mitochondria from one species to mutants lacking mitochondrial DNA (ρ0) from a second species, according to the restoration of the ability to respire and grow on a non-fermentable carbon source. In early studies mitochondria from unrelated yeast species were introduced to S. cerevisiae (Yoshida, 1979; Sakanaka et al., 1996; Osusky et al., 1997). However, these experiments could not be reproduced (Špírek et al., 2000) and most of them should be considered experimental artifacts, mainly as consequence of the absence of mtDNA analysis in cybrids and unreliable taxonomy, as most of the “unusual” compatible yeasts were misclassified (Barnett, 1992; Špírek et al., 2000; Vaughan-Martini and Martini, 2011; Sulo, unpublished results). Currently, the Saccharomyces genus consists of the species S. cerevisiae, S. paradoxus, Saccharomyces mikatae, Saccharomyces kudriavzevi, S. arboricolus, S. bayanus var. bayanus, S. bayanus var. uvarum, and Saccharomyces pastorianus (Vaughan-Martini and Martini, 2011). However, whole genome analysis has shown that the last three species are cold-adapted allopolyploids of S. cerevisiae and the newly described species Saccharomyces eubayanus (reviewed in Hittinger, 2013). In addition, Saccharomyces cariocanus is considered to be more a S. paradoxus variant as this species is reproductively isolated from S. paradoxus by four translocations but not by sequence (Liti et al., 2006; reviewed in Hittinger, 2013). Consequently the successful re-establishment of mitochondrial functions in S. cerevisiae ρ0 strains has been achieved by the transplacement of isolated mitochondria or protoplast fusion only from yeasts currently classified as synonymous to S. cerevisiae (Osusky et al., 1997; Špírek et al., 2000) or the most closely-related species S. paradoxus (Špírek et al., 2000). However, in this case the rate of the re-established respiration was only partial (Špírek et al., 2000). In the opposite direction, S. cerevisiae mtDNA was able to restore respiration in S. paradoxus mitochondria to a level close to the original (Sulo et al., 2003), indicating the unidirectional character of these nucleo-mitochondrial incompatibilities (Sulo et al., 2003), a phenomenon that has been confirmed in interspecific hybrids with different combinations of nuclear and mitochondrial genomes (Chou et al., 2010).

Thus, xenomitochondrial cybrids are a valuable tool in the study of nucleo-mitochondrial interactions, particularly those determining the primary divergence of biological species. In this work we elaborated a simple procedure for the preparation of xenomitochondrial cybrids with the aim to report nucleo-mitochondrial incompatibilities associated with an inability to splice an unusual mitochondrial intron coxI138 and the capability of cells to compensate for the splicing defect after the adaptation period by the gain-of-function of nuclear genes.

**MATERIALS AND METHODS**

**YEAST STRAINS**

The Saccharomyces species used for the transfer of mitochondria to S. cerevisiae were as follows: S. cerevisiae NRRL Y-12632T (CBS 1171), S. paradoxus NRRL Y-17217T (CBS 432), S. paradoxus (CBS 2908), S. kudriavzevi NRRL Y-27339T (CBS 8840), S. cariocanus NRRL Y-27337T (CBS 7994), S. mikatae NRRL Y-27341T (CBS 8839), S. bayanus var. uvarum NRRL Y-17034T (CBS 395), S. bayanus var. bayanus NRRL Y-12624T (CBS 380), and S. pastorianus (CBS 1504, CBS 1513), which were obtained from J. Piškúr Lund University. The following abbreviations apply: CBS corresponds to the Culture Collection of the Centraalbureau voor Schimmelcultures and Fungal Biodiversity Center, Utrecht, The Netherlands; NRRL to the Agricultural Research Service Culture Collection, US Department of Agriculture, Peoria, Illinois, USA; andNCYC to the National Collection of Yeast Cultures, Institute of Food Research, Norwich, United Kingdom. The superscript T in yeast designations indicates type of strains (Vaughan-Martini and Martini, 2011). The other S. cerevisiae wild type strains used in this study are as follows: CCY 21-37-2 (CBS 457), CCY 21-46-1 (CBS 4054), CCY 21-37-1 (CBS 1426), CCY 21-45-1 (CBS 1782), CCY 21-8-1 (CBS 436), CCY 21-4-11 (CBS 1460), CCY 21-11-1 (formerly Saccharomyces chevalieri, CBS 400), CCY 21-10-1 (CBS 435), CCY 21-36-1, CCY 21-42-1 (formerly Saccharomyces

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capensis, CBS 2247), CCY 21-33-3 (formerly Saccharomyces italica-cus, CBS 2909), CCY 21-21-1 (formerly Saccharomyces oviformis, CBS 429), CCY 21-14-1, CCY 21-1-1 (CBS 382), CCY 21-4-27 (CBS 1200), CCY 21-15-6 (CBS 439), and CCY 21-15-1 (CBS 381), which were obtained from the Culture Collection of Yeasts (CCY; the former Czechoslovak Collection of Yeasts), located at the Institute of Chemistry, Slovak Academy of Sciences, in Bratislava. The proper taxonomic classification of any strain used in this study has been confirmed by the sequencing of the D1/D2 region from a large ribosomal RNA subunit and mitochondrial cox2 and rns genes, as described in Kurtzman (2003). S. paradoxus (synonymous to Saccharomyces douglasii) CBS 7400 strain was kindly provided by H. Fukuhara, Institute Curie, Orsay. The laboratory acceptor strains of mitochondria MCC109 \( \rho^0 \) (\( \text{MAT} \alpha, \text{ade}2-1, \text{ura}3-52, \text{kar}1-1, \rho^0 \)); mit\(^−\) testers PTY6 (\( \text{MAT} \alpha, \text{ade}2, \text{ura}3-52, \text{kar}1-1, \text{cox}2-17 \)), K2145 (\( \text{MAT} \alpha, \text{ade}2, \text{lys}, \text{kar}1-1, \text{cox}2-17 \)), 2612 [\( \text{MAT} \alpha, \text{ade}1, \text{op}1, \text{mit}^- (\text{cox}1 \Delta B) \)], MD 79 [\( \text{MAT} \alpha, \text{lea}1, \text{kar}1-1, (\text{cox}1-\Delta \text{delB}) \)], akarl170 [\( \text{MAT} \alpha, \text{his}^-, \text{lys}^-, \text{kar}1-1, \text{sym}^- \) (\( \text{trd} \) C → A (72)], V281 [\( \text{MAT} \alpha, \text{ade}1, \text{op}1, \text{mit}^- (\text{cox}1, \text{a}11 \text{T} \rightarrow \text{A}(1856)) \)], aOP1/M1301 [\( \text{MAT} \alpha, \text{op}1, \text{ura}3-1, \text{leu}2-3, 112, \text{his}3-11,15, \text{trp}1-289, \text{mit}^- (\text{cox} b11M1301) \)], as well as derivatives of W303 IA (\( \text{MAT} \alpha, \text{ade}2-1, \text{trp}1-2, \text{leu}2-3, 112, \text{his}3-11,15, \text{ura}3-1, \text{can}1-100, \text{Gal}^+, \text{psi}^+ \), \( \rho^+ \)), DBY 747 (\( \text{MAT} \alpha, \text{trp}1-289, \text{leu}2-3, 112, \text{his}3-1, \text{ura}3-52, \text{Gal}^-, \text{mit}^- (\text{cox} b11M1301) \)) have been described by Špírek et al. (2000, 2002) and Sulo et al. (2003). Other mit\(^−\) strains 5B [\( \text{MAT} \alpha, \text{ade}1^-, \text{lys}^-, \text{mit}^- (\text{cox}1 \text{ai}1 \text{ai}2 \text{junction}) \)], and AD1 [\( \text{MAT} \alpha, \text{ade}1^-, \text{lys}^-, \text{mit}^- (\text{cox}1 \text{ai}1 \text{ai}2 \text{junction}) \)] are described in detail in Anziano et al. (1990).

**Media**

Yeasts were routinely cultivated on YPD (1% bactopeptone, 1% yeast extract, 2% glucose), YPGE (1% bactopeptone, 1% yeast extract, 3% glycerol, 2% ethanol), minimal medium (0.67% YNB, 2% glucose), and sporulation media (0.1% yeast extract, 1% potassium acetate, 0.05% glucose).

**Transplacement of mitochondria by interspecific cytoduction**

Sporulated diploid collection yeasts (10⁸–10⁹/50μl) were treated with Zymolyase 20T 0.5 mg/ml. After 10–20 minutes (when the majority of spores were released), the spores were vortexed for 30 s and washed with distilled water mixed with a twofold amount of fresh MCC109 \( \rho^0 \) cells, and the suspension was applied in a small volume (10–50 μl) on YPD plates. After one day’s incubation the mating efficiency was controlled microscopically and aliquots of 10⁸ cells were streaked onto selection FOA plates (3% glycerol, 2% ethanol, 0.5% glucose, 0.67% YNB, 20 mg/l adenine, 50 mg/l uracil, 2% agar, with 1 g/l of 5-fluoroorotic acid AUDFOA) for the selection of respiring cybrids (2% glucose, 0.67% YNB, 20 mg/l adenine, 50 mg/l uracil, 2% agar, with 1 g/l of 5-fluoroorotic acid AUDFOA) to select any cybrids. The plates were incubated at 28°C and after 5–7 days they were scraped and resuspended in demineralized water and spotted in tenfold dilutions onto YPD plates. After 3 days of cultivation the number of colonies grown from single drops were counted and plated in corresponding dilutions on AUDFOA or YPD plates. Single colonies were then screened for correct auxotrophic markers and the ability to grow on YPGE plates. The presence of mtDNA in colonies that did not grow on YPGE plates was determined by restriction digestion or after mating to the mit\(^−\) tester strain K2145, allowing non-adapted or non-respiring cybrids to be studied. A similar selection procedure is widely used when DNA is delivered to mitochondria by biolistics (Fox et al., 1991). The approach has a few constraints, such as the poor sporulation ability observed in a number of the collection strains. However, the problem can be bypassed by several cycles of sporulation and germination. The other expected pitfall, low mating ability, was not observed since even the less related species S. cerevisiae and S. bayanus produced an incredible amount of zygotes (Marinoni et al., 1999). Finally, the acceptor strain lacking mtDNA must carry the kar1-1 mutation, because their substitution by regular \( \rho^0 \) strains does not yield cybrids but hybrids.

**DNA analysis**

The presence of mtDNA in cybrids was confirmed by the Hin\(f\)I restriction digestion of genomic DNA isolated according to the laboratory modification of Philipseps et al. (1991). Mitochondrial DNA was isolated according to Defontaine et al. (1991). Karyotypes have been determined by pulsed-field electrophoresis, as described in Marinoni et al. (1999), with minor modification. Electrophoresis was carried out at 150 V for 6.5 h, with a switching time of 240 s followed by 8 h with a switching time of 160 s, then 9 h 45 minutes with pulse duration of 90 s, and finally 14 h 38 minutes with 60 s pulse duration.

**DNA amplification and sequencing**

D1D2 domain of 26S rDNA was amplified according to Kurtzman and Robnett (2003), part of the mitochondrial cox2 gene was amplified with primers cox2 357: 5’-CAG GAT CCA GCA ACA CCA AAT CAA GA and cox2 rev: 5’-CAT GCC CCA TAG AAG ACA CTT TCT CT (amplification condition 94°C – 3 min, 35 × (94°C – 30 s, 52°C – 1 min, 72°C – 1 min), 72°C – 5 min, 14°C). Part of rrs gene was amplified with primers YM5: 5’-AAG AAT ATG TTG GTT CAG A and YM13: 5’-ATT CTA CGG ATC CTT TAA ACC A (amplification condition 94°C – 3 min, 35 × (94°C – 30 s, 45°C – 1 min, 72°C – 2 min), 72°C – 5 min). CoxI3B was amplified using the primers SDA2: 5’-AAT CTA CAC TAG GTG TTG GTG GAT AAC CTA CTG ACT (amplification condition 94°C – 3 min, 35 × (94°C – 30 s, 45°C – 1 min, 72°C – 2 min), 72°C – 5 min). Entire cox1 mtDNA sequences were obtained as described in Prochazka et al. (2012) or were extracted from running the whole mtDNA sequencing project using Illumina MiSeq and paired-end (2 × 100 nt technology) assembled with NCBI genomes Workbench 7.0.3 (http://www.ncbi.nlm.nih.gov).

**RNA analysis**

RNA was isolated as described by Köhler and Domdey (1991). Thirty micrograms was separated on 1.0% agarose–formaldehyde gels, transferred to a nylon membrane, and hybridized by the DIG High Prime DNA Labeling and Detection Kit (Roche Molecular Biochemicals) labeled probes, as described in Brown (1993).
Disruption of MRP13 gene
The region between BamHI and XbaI restriction site of the gene was replaced with a LEU2 gene XbaI-BamHI fragment and introduced to the haploid spores of adapted and non-adapted cybrids carrying the leu2 mutation and S. paradoxa CBS 7400 mtDNA as well as to W303 1A ρ0 and ρ+ strains. Disruption was confirmed after Southern blotting by hybridization with MRP13 specific probe.

Oxygen consumption
Oxygen consumption was measured at 30°C with a Clark oxygen electrode using 2% ethanol as the substrate. Cybrid cells were harvested and washed twice with distilled water. Oxygen consumption was measured in 2 ml water suspension with 2% ethanol as a substrate. Respiratory rates were expressed as consumed nmoles O2/min per 5 x 10^8 cells. The solubility of the O2 237 nmol/ml water was considered and at the end of measurement oxygen consumption was inhibited by the addition of antimycin at the final concentration of 10 μg/ml.

Cytochrome spectra
Cytochrome spectra were recorded in mitochondrial suspensions (0.4 M sorbitol, 5 mM EDTA pH 6–8, 0.25% BSA 8 mg/ml of proteins) by Double Wavelength Double Beam Perkin-Elmer 557 spectrophotometer, as described in Tzagoloff et al. (1975). Mitochondria were isolated according to (Sulo et al., 1989) from an overnight culture grown on 1% bactopeptone, 1% yeast glucose) on the plates with a non-fermentable carbon source. FOA is lethal for any yeast carrying the wild type URA3 gene (Sikorski and Boeke, 1991), and therefore it inhibits the growth of “true hybrids” and all wild type Saccharomyces strains (Figure 1). Cybrids with various degrees of re-established mitochondrial functions can be also obtained on synthetic selection plates with FOA and glucose as a carbon source, as they can preferentially survive 5–7 days incubation (Figure 1B). After plating to a single colony, cybrids from donor species can be distinguished according to their auxotrophy.

The presence and origin of mitochondrial genomes can be unambiguously assigned according to restriction fragment polymorphisms that have long been used for molecular “typing” of different strain-to-strain variations in yeasts (e.g., Querol et al., 1992; Spírek et al., 2000). Consequently HinI or EcoRV restriction profiles of all the cybrids chosen for further study were identical to the mtDNA profiles from parent species, which excludes the rearrangement of DNA (Figures 2A–C). The authenticity of transferred mt genomes was confirmed also by whole genome sequencing. The very rare event of single chromosome transfer is known if FOA selection pressure is applied (Nilsson-Tillgren et al., 1980), and this could possibly be responsible for compatibility. Cybrid karyotypes were not distinguishable from the original respiration deficient S. cerevisiae strain but variable enough to distinguish hybrids with both genomes and some of single chromosome transfers (Figure 2D; Marinoni et al., 1999). The uniform karyotype excludes the heterokaryon variants and the transmission of intact nuclei from the mitochondrial donors. Auxotrophic phenotypes and mating type were well-preserved in all examined cybrids.

RESULTS
THE ABILITY TO POPULATE S. CEREVISIAE WITH MITOCHONDRIA FROM RELATED SPECIES—PHYLOGENETIC LIMIT
To elucidate the limit of nucleo-mitochondrial compatibility among Saccharomyces, we made an attempt to construct xenomitocondrial cybrids containing nuclei from S. cerevisiae and mtDNA from all other Saccharomyces species. To avoid any misinterpretations resulting from incorrect taxonomic classification, the origin of any strain used in this study has been verified by the sequencing of the D1/D2 region from the large ribosomal RNA subunit and mitochondrial cox2 and rrs genes, as described in Kurtzman (2003). The collection consisted of about 20 S. cerevisiae, three S. paradoxa, strains, of which two were involved in a previous study (Spírek et al., 2000), and three relatively new but quite well-characterized species: S. cariocanus, S. kudriavzevii, and S. mikatae (Naumov et al., 2000) and S. bayanus. Instead of protoplast fusion (Spírek et al., 2000) the mitochondria were transferred by mating the spores from different Saccharomyces species to ade2-1, ura3-52, kar1-1, ρ0 S. cerevisiae strain, since the kar1-1 mutation in the acceptor strain significantly reduces the karyogamy events (Fox et al., 1991). Cybrids regaining efficient mitochondrial functions can be simply selected as the mostly red/pink colonies on the plates containing 5-fluoroorotic acid (FOA) and an excess of non-fermentable carbon source. FOA is lethal for any yeast carrying the wild type URA3 gene (Sikorski and Boeke, 1991), and therefore it inhibits the growth of “true hybrids” and all wild type Saccharomyces strains (Figure 1). Cybrids with various degrees of re-established mitochondrial functions can be also obtained on synthetic selection plates with FOA and glucose as a carbon source, as they can preferentially survive 5–7 days incubation (Figure 1B). After plating to a single colony, cybrids from donor species can be distinguished according to their auxotrophy.

THE RE-ESTABLISHMENT OF NUCLEO-MITOCNDRIAL COMPATIBILITY WITH FOREIGN mtDNA IN S. CEREVISIAE OFTEN ACCOMPANIES THE ADAPTATION PROCESS
Cybrids with partially re-established mitochondrial functions can be obtained on selection media containing glucose. If cells from a single cybrid colony are transferred from selection media (with glucose) on the plates with a non-fermentable carbon source such as glycerol and ethanol (YPGE), unambiguously good growth is displayed only in cybrids containing mitochondrial genome from various S. cerevisiae strains, the S. paradoxa strain CBS 432, and the S. cariocanus strain CBS 7994 (Figure 3, Table 1). Other cybrids gave rise to a mixed population that consisted mainly of poorly growing colonies. However, most of these cybrids with
FIGURE 1 | Construction of xenomitochondrial cybrids. (A) General selection chart. (B) Plating of cytoduction mixture MCC109 ρ0 × S. kudriavzevii CBS 8840 after 5 days cultivation on selection medium with FOA on YPD plate. Detail: smaller white colonies – non-adapted cybrids; larger pink colonies – adapted cybrids.

mtDNA from S. paradoxus CBS 7400, S. paradoxus CBS 2908, and S. mikatae CBS 8839 were capable of forming colonies on non-fermentable substrate after prolonged incubation on YPGE (Figure 3, Table 1), indicating an adaptation process. Cybrids with mt genome S. kudriavzevii CBS 8840 growing on glycerol also appeared, with a shorter but significant delay. Even after 2 months of incubation we did not observe any colony growing on glycerol among the cybrids containing mtDNA from the less-related yeasts of the S. bayanus – uvarum group.

To understand the nature of the adaptation process, we transferred the cytoplasm from adapted cybrids capable of growing on YPGE again by cytoduction to a different S. cerevisiae W303 1A ρ0 strain (carrying YEp352 plasmid with URA3 selection marker) in a ratio of 100:1. Putative cytoductant colonies were selected on glucose plates lacking uracil, and the presence of mtDNA was determined by DAPI staining. Colonies with W303 1A auxotrophic markers containing mtDNA were then transferred onto rich YPGE plates, and their growing ability was examined (Table 1). Again, the ability to utilize a non-fermentable carbon source in these second generation cybrids mimicked the behavior of cybrids obtained by first transfer from parental non-cerevisiae species. In spite of the reduced adaptation period, the proportionality confirms that transfer of nuclear genes from original parental species is not required.

Reduced spore viability, used in taxonomic classification, is a very profound feature of interspecific yeast hybrids as well the landmark of nucleo-mitochondrial Dobzhansky–Muller incompatibility (Naumov et al., 2000; Chou and Leu, 2010). Therefore, S. cerevisiae cybrids repopulated with foreign mtDNA and capable of growing on a non-fermentable carbon source (adapted) were crossed with the S. cerevisiae W303 1A ρ0 strain carrying the YEp352 plasmid. Diploids were selected on the minimal media supplemented with adenine, and their sporulation ability was examined. All diploids could sporulate and the majority of spores (from 70 to 90%) germinated, suggesting the absence of Dobzhansky–Muller incompatibility pairs. In addition, spores from asci were separated by micromanipulator and full tetrads were tested for auxotrophic requirements and the ability to grow on YPGE. The segregation of the ability to grow on glycerol–ethanol when the mating partner was cybrid harboring mtDNA from S. paradoxus CBS 432, S. cariocanus CBS 7994, or any S. cerevisiae was at a ratio of 4:0, which emphasizes the direct compatibility of mitochondrial and nuclear genomes from different species. However, if one of the mating partners was a cybrid with mtDNA from S. paradoxus CBS 2908, S. paradoxus CBS 7400, S. mikatae CBS 8839, or S. cariocanus CBS 7994, the ability to grow on glycerol and ethanol segregated at a 2:2 ratio (Figure 4), indicating a single gene gain-of-function, responsible for the adaptation phenomenon. In the case of cybrids with S. kudriavzevii CBS 8840 mtDNA, the ratio was unexpectedly 3:1, although a small number of tetrads segregated at a ratio of 2:2 or 4:0, suggesting most likely the gain-of-function in two different genes involved in the adaptation process. The authenticity of the tetrads was always confirmed by the segregation of nuclear markers.

EFFECTIVENESS OF OXIDATIVE PHOSPHORYLATION IN THE XENOMITOCHONDRIAL CYBRIDS

The performance of oxidative phosphorylation was assessed according to the rate of oxygen consumption, measured in cultures grown in liquid YPD medium followed by a 12 h cultivation in YPGE, which allows non-adapted variants to be compared as well. The respiration capacity of cybrids that display good nucleo-mitochondrial compatibility (compatible mt genomes) from the S. paradoxus strain CBS 432 and S. cariocanus CBS 7994 decreased to 40% of the S. cerevisiae (Table 2). The oxygen consumption was reduced to 20–30% in adapted cybrids with mtDNA from S. kudriavzevii, S. paradoxus CBS 7400, S. paradoxus
CBS 2908, and S. mikatae CBS 8839. A threshold respiration rate about 2–10% of the wild type can be detected in non-adapted variants but not in a cybrid with S. bayanus mtDNA.

The growth rate on the medium with non-fermentable carbon is widely used as a good criterion for assessing the phenotype of various mitochondrial mutations. Figure 5A shows that cybrids with mitochondria from the S. paradoxus strain CBS 432 and S. cariocanus CBS 7994 grow nearly at the same rate as cybrids with S. cerevisiae mitochondria. Cybrids requiring an adaptation period (S. paradoxus CBS 7400, S. paradoxus CBS 2908, S. mikatae CBS 8839) exhibit a significantly slower growth rate. Besides the slower growth rate, they exhibit an extremely long lag phase (3–8 days) if they are transferred from glucose to glycerol (Figure 5B).

ADAPTATION INTRODUCES TEMPERATURE SENSITIVE PHENOTYPE ON GLUCOSE

To shed more light on the scale of the communication ability of xenomitochondrial cybrids, we examined their ability to grow at an elevated temperature. While cybrids with mtDNA from S. cerevisiae and compatible Saccharomyces strains (S. paradoxus CBS 432 and S. cariocanus CBS 7994) were capable of growing at 37°C, even on non-fermentable carbon sources, other cybrids with non-cerevisiae mtDNA were not. Surprisingly, they were not capable of growing at an elevated temperature even on YPD with glucose as a carbon source, while the parental S. cerevisiae ρ₀ mutant was. Their growth was abolished even after the transfer from 37 to 30°C, indicating the lethal effect of elevated temperature (Figure 6).

MITOCHONDRIAL cox1 GENE IS THE MAIN DETERMINANT OF XENONUCLEO-MITOCHONDRIAL COMPATIBILITY

The segment of mtDNA responsible for interspecific incompatibility can be determined by petite mapping (Fox et al., 1991). Therefore, we first crossed all non-adapted cybrids with S. cerevisiae mit − strains carrying mutations in single mitochondrial genes cox1, trnD, cob, and cox2 (details listed in the section Materials and Methods). All combinations grew well on YPGE plates, but not diploids from the crosses of three different cox1 mutants (carrying deletion of exon2 AD1, 5B or 8.2 kb deletion of exon2–intron 15θ MD 79) suggested an impaired compatibility associated with the cox1 gene (Figure 7). This outcome was confirmed by petite mapping using ρ − mutants.
prepared from the *S. cerevisiae* DBY 747 strain harboring intronless mtDNA (Seraphin et al., 1987). All ρ<sup>-</sup> strains able to complement the growth defects of non-adapted cybrids carried mtDNA fragments containing the entire *coxl* gene, which was confirmed by genetic and physical mapping.

A defect in the large subunit of the *coxl* gene confirmed cytochrome spectra of reduced vs. oxidized mitochondrial extracts (Tzagoloff et al., 1975). The ratio of cytochromes *aa3* similar to the wild type is maintained in extracts (Tzagoloff et al., 1975). The ratio of cytochromes *aa3* peak is barely detectable in non-adapted forms and reaches about 50% of the wild level after adaptation. In *S. cerevisiae* with *S. bayanus* mtDNA the cytochrome *aa3* signal is missing.

It appears that the defect in *coxl* expression is a major drawback in the interspecific nucleo-mitochondrial (in)compatibility in *Saccharomyces* (Figure 8).

### Table 1 | Re-established growth on non-fermentable carbon source (YPGE).

| *S. cerevisiae* cybrids (MCC109 ρ<sup>0</sup>) with mitochondrial genome from: | Proportion of colonies growing immediately on YPGE in (%)<sup>b</sup> | A period required for re-established growth on YPGE in (days)<sup>b</sup> | A period required for re-established growth on YPGE in (days)<sup>b</sup> after second cytoduction to W303 1A strain<sup>d</sup> |
|---|---|---|---|
| *S. cerevisiae* W303 1A | 100 | 0 | 0 |
| *S. cerevisiae* CBS 400<sup>a</sup> | 100 | 0 | 0 |
| *S. paradoxus* CBS 432 | 100 | 2 | 6–14 |
| *S. paradoxus* CBS 2908 | 2 | 6–14 | 4 |
| *S. paradoxus* CBS 7400 | 2 | 6–14 | 4 |
| *S. kudriavzevi* CBS 8840 | 5 | 1–3 | 1 |
| *S. mikatae* CBS 8839 | 15 | 6–14 | 4 |
| *S. cariocanus* CBS 7994 | 100 | 0 | 0 |
| *S. bayanus* CBS 380 | 0 | >60 | – |
| *S. cerevisiae* MCC109 ρ<sup>0</sup> | 0 | >60 | – |

<sup>a</sup>The same was observed for other *S. cerevisiae* wild type strains listed in the Section Material and Methods and any examined respiring laboratory strain.

<sup>b</sup>Up to 50 colonies were examined.

<sup>c</sup>As visible colonies that arose from patch.

<sup>d</sup>Mitochondria transferred from primary cybrids to the strain W303 1A ρ<sup>0</sup> transformed with the plasmid YEp352 by kar cross.

### Table 2 | Respiration capacity of cybrids.

| *S. cerevisiae* cybrids (MCC109 ρ<sup>0</sup>) with mitochondrial genome from: | Respiration capacity (nmol O<sub>2</sub>/min/5 × 10<sup>8</sup> cells) | Relative respiration capacity (%) |
|---|---|---|
| *S. cerevisiae* W303 1A | 190 ± 50 | – |
| *S. cerevisiae* CBS 400 | 180 | – |
| *S. paradoxus* CBS 432 | 90 | – |
| *S. cariocanus* CBS 7994 | 80 | – |
| *S. kudriavzevi* CBS 8840 | 80 | – |
| *S. paradoxus* CBS 7400 | 77 | – |
| *S. paradoxus* CBS 2908 | 64.6 | – |
| *S. mikatae* CBS 8839 | 43.7 | – |
| *S. bayanus* CBS 380 | 55 | – |
| *S. cerevisiae* MCC109 ρ<sup>0</sup> | 64 | – |

<sup>A</sup>adapted; <sup>NA</sup> non-adapted.

**FIGURE 4** | Adaptive gain-of-mutation is linked to nuclear gene.

Zygotes from mating of adapted cybrids with *S. paradoxus* CBS 7400 mtDNA and W303 1A ρ<sup>0</sup> strain were sporulated and tetrads dissected. Colonies from individual spores were spotted on different media. (3–6) Four individual tetrads; Controls: (1) MCC109 ρ<sup>0</sup>; (2) cybrid with *S. paradoxus* CBS 7400 mtDNA; (7) W303 1A ρ<sup>+</sup>; (8) W303 1A ρ<sup>0</sup>.

Plates (A) YPD 30°C; (B) YPD 37°C; (C) YPGE 37°C; (D) YPGE 30°C. Cultivated 3 days at 28°C.
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**FIGURE 5 | Growth rates of adapted cybrids.** Cells grown in liquid complete medium with glycerol and ethanol (YPGE). (A) Inoculum from YPGE; (B) inoculum from YPD, *S. cerevisiae* cybrids (strain MCC109 ρ0) with mt genomes from: *S. cerevisiae* strain W303 1A (●), *S. bayanus* CBS 380 (○); *S. paradoxus* CBS 432 (■); *S. cariocanus* CBS 7994 (□); 1 OD600 unit equals 6 × 10⁷ cells/ml.

**FIGURE 6 | Temperature sensitive growth of xenomitochondrial cybrids.** Cultivation (A) YPD 30°C, (B) YPD 37°C (C) YPGE 30°C, (D) YPGE 37°C: *S. cerevisiae* cybrids with mitochondrial genomes from *S. cerevisiae* strain W303 1A (1); *S. paradoxus* CBS 432 (2); *S. paradoxus* CBS 2908 (3); *S. kudriavzevii* CBS 8840 (4); *S. mikatae* CBS 8839 (5); *S. paradoxus* CBS 7400 (6); *S. cariocanus* CBS 7994 (7).

**FIGURE 7 | Cox1 gene as the main determinant of xenonucleo-mitochondrial (in)compatibility.** (I) Growth of non-adapted *S. cerevisiae* cybrids with mitochondrial genomes from *S. paradoxus* CBS 7400 (A), *S. paradoxus* CBS 2908 (B), *S. mikatae* CBS 8839 (C), *S. kudriavzevii* CBS 8840 (D), *S. bayanus* CBS 380 (E), MCC109 ρ0. Strain (negative control) (F); after mating to different mt− strains: (1) MD79 (mt− in cox1), (2) akar170 (mt− in trnAsp), (3) M1301 (mt− in cob), (4) AD1 (mt− in cox1); (5) 5B (mt− in cox1); (6) K2145 (mt− in cox2). Cultivated on YPGE plates for 4 days at 30°C. Growth of non-adapted cybrids after the mating with *S. cerevisiae* DBY 747 ρ0 (II) and *S. cerevisiae* DBY 747 ρ− containing intronless cox1 gene (III) mt− strain 2612 (positive control) (1); *S. cerevisiae* cybrids with mitochondrial genomes from: *S. paradoxus* CBS 7400 (2); *S. paradoxus* CBS 2908 (3); *S. kudriavzevii* CBS 8840 (4); *S. mikatae* CBS 8839 (5); *S. paradoxus* CBS 7400 (6). Plates (A) YPD 30°C, (B) YPD 37°C, (C) YPGE 30°C, (D) YPGE 37°C. Cultivation 4 days.
mRNA about 1700 nt long, and the second was linked to longer mRNA 2500 nt in size, corresponding to the coxl premRNA with unspliced I3β intron RNA (Figure 9A). The signal intensity of longer form was very profound in cybrids, but was noticeable in parental strains only after overexposure. The membrane was stripped and hybridized with a probe specific to first two exons from S. cerevisiae coxl gene. (A) Lanes: S. cerevisiae (W303 1A $ρ^+$) (1); S. paradoxus CBS 432 parent (2), cybrid (3); S. kudriavzevi CBS 8840 parent (4), cybrid (5); S. paradoxus CBS 2908 parent (6), cybrid (7); S. paradoxus CBS 7400 parent (8), cybrid (9); S. mikatae CBS 8839 parent (10), cybrid (11); S. cerevisiae MCC109 $ρ^+$ (12). (B) The same membrane stripped and hybridized with probe specific to I3β from S. paradoxus CBS 7400. Markers: 3360 nt 26S rRNA; 1710 nt 18S rRNA.

FIGURE 9 | Northern blot of RNA from parental species and adapted cybrids. RNA separated on agarose–formaldehyde gel, transferred on nylon membrane was hybridized with the probe specific to first two exons from S. cerevisiae coxl gene. (A) Lanes: S. cerevisiae (W303 1A $ρ^+$) (1); S. paradoxus CBS 432 parent (2), cybrid (3); S. kudriavzevi CBS 8840 parent (4), cybrid (5); S. paradoxus CBS 2908 parent (6), cybrid (7); S. paradoxus CBS 7400 parent (8), cybrid (9); S. mikatae CBS 8839 parent (10), cybrid (11); S. cerevisiae MCC109 $ρ^+$ (12). (B) The same membrane stripped and hybridized with probe specific to I3β from S. paradoxus CBS 7400. Markers: 3360 nt 26S rRNA; 1710 nt 18S rRNA.

FIGURE 8 | Cytochrome spectra of parental species and xenomitochondrial cybrids. Absorption maxima for cytochrome aa$_3$ – 603 nm; cytochrome b – 560 nm; cytochrome $c_1$ – 550 nm. Abbreviations: P: parent; C: cybrid; A: adapted cybrid; N: non-adapted cybrid.

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can be compensated for by gain of function that involves multiple genes.

**DISCUSSION**

*S. cerevisiae* xenomitochondrial cybrids can be prepared simply by the mating of ρ0 strains with impaired karyogamy and germinating spores of different *Saccharomyces* taking advantage of the *ura3* mutation and fluororotic acid selection. Cytoduction allows the construction of cybrids that grow poorly if at all on a non-fermentable carbon source due to their ability to survive the prolonged incubation on solid synthetic media with FOA better.

In general, all *S. cerevisiae* xenomitochondrial cybrids fall into three categories. The first includes cybrids containing mitochondrial genome from other *S. cerevisiae* strains, and two different species *S. paradoxus* CBS 432 and *S. cariocanus* CBS 7994 (Table 2). Besides a decreased respiration rate, the last two cases do not exhibit any stronger phenotype. They are metabolically and genetically similar to cybrids with mitochondrial genomes from different *S. cerevisiae* strains. Their growth rate, cytochrome content, and respiration rate is slightly lower, and they also display a short lag phase extension after the shift from glucose to glycerol-ethanol media. Cytoduction experiments and the segregation of glycerol grown phenotype in a 4:0 ratio in tetrads after mating to *S. cerevisiae ρ0* confirmed that these two mtDNA do not require changes in any nuclear gene to re-establish usable oxidative phosphorylation. Mismatched mitochondrial and nuclear genomes from two different *S. cerevisiae* strains often result in very profound reduced fitness on non-fermentable medium (Zeyl et al., 2005; Dimitrov et al., 2009; Palival et al., 2014). It is difficult to decide whether minor incompatibilities in this class of compatible cybrids are due to nucleo-mitochondrial epistasis or whether some “interspecific” factor may play a role.

The second class consists of cybrids containing mtDNA from two other *S. paradoxus* strains, *S. mikatae* and *S. kudriavzevii*, which exhibit an impaired performance of oxidative phosphorylation and a poor ability to grow on non-fermentable substrate after the transplacment of mitochondria. However, long-term incubation on glycerol media that may last for several weeks initiates the growth of adapted variants. Their respiration capacity is as low as 2–3% and increases to 20–30% of wild type level due to the elevated cytochrome *aa3* content resulting from improved *cox1-13β* intron splicing. This period of adaptation feature has not been observed in any other well-characterized xenomitochondrial cybrids from primate or rodent cells, and it seems to be yeast specific (Dey et al., 2000; McKenzie and Trounce, 2000; Yamaoka et al., 2000; Burton et al., 2013). The capability of growing on glycerol is temperature sensitive and this gain-of-function is associated with the loss of the ability to grow at 37°C even on glucose, a fermentable substrate that can be utilized anaerobically. With the exception of cybrid with *S. kudriavzevii* and *S. paradoxus* CBS 432 mtDNA, the exposure to an elevated temperature of 37°C is lethal and cells do not grow after replica-plating on a fresh YPD plate. They also exhibit a slower growth rate on glycerol, and a very remarkable feature is the extremely long lag phase after the shift from glucose to glycerol–ethanol media (Figure 5B). They appear to behave like nucleo-mitochondrial communication mutants. We failed to find any similar phenotype from the literature search, but temperature sensitive mutations in general indicate defects in the protein-coding genes often essential for cell viability (Hampsey, 1997). Conditionally, lethal growth on a non-fermentable carbon source is quite frequent and has been reported as a consequence of deletion/mutation in a few genes, for example, *ABF2* coding for HMG-like DNA binding protein (Kao et al., 1993) and *MMF1* for protein with unknown function, but present in eukaryotes and prokaryotes (Oxelmark et al., 2000); however, these mutants are capable of growing at an elevated temperature on glucose. The respiration capacity of adapted cybrids is sufficient to perform a normal yeast cell cycle. They can sporulate after mating to *S. cerevisiae ρ0* strains and tetrad analysis has demonstrated their ability to grow on glycerol segregated mostly at a 2:2 ratio. The most plausible explanation for this segregation is some unspecified adaptive gain-of-mutation(s). After prolonged cultivation, single colonies arose from the patches of originally non-growing spores on glycerol media, which underlines their ability to adapt under selection pressure. A similar “adaptation” phenotype has been reported for mutants lacking

**FIGURE 10** The ability of non-adapted cybrids transformed with xenoadjustors to grow on non-fermentable carbon source.

*S. cerevisiae* cybrids with mitochondrial genomes from (A) *S. bayanus* CBS 380 (B) *S. paradoxus* CBS 2908 (C) *S. paradoxus* CBS 7400 (D) *S. mikatae* CBS 8839 (E) *S. kudriavzevii* CBS 8840 transformed with plasmid containing genes: 1 – none (pYES2), 2 – *MRP13*, 3 – *MRS2*, 4 – *MRS3*, 5 – *MRS4*, 6 –none (YPE24). Cultivation 4 days.
mitochondrial porin. Deletion of the gene impairs some respiratory functions and therefore the growth on non-fermentable carbon sources; however, after a lag phase porinless mutant cells adapt to growth on glycerol (Dihanich et al., 1987). Apparently, this type behavior is not exceptional if the introduction of foreign mtDNA into \textit{S. cerevisiae} is considered as the mutation. In yeast cells, such intervention puts pressure on the organism’s genome to compensate for it so that it leads to a mutation in other genes (Teng et al., 2013).

Northern blot revealed the limited ability of \textit{cox1}-I3β to splice as the most plausible explanation of the different behavior observed in these two cybrid classes. We made much effort to obtain the clean elimination of \textit{cox1}-I3β by cultivation of cybrids at 37°C. This should have introduced selection pressure that resembles intron mutation, principally used in the preparation of intronless mtDNA strain (Seraphin et al., 1987). However, \textit{cox1}-I3β intronless variants were not obtained in spite the fact that two of transferred genomes carried group II introns needed for in vivo deletion of mitochondrial introns (Figure 11: Levra-Juillet et al., 1989). Also, we tried to eliminate the intron by mating with appropriate petites, but several tens of petite strains capable of complementing the glycerol growth defect harbored a much longer \textit{cox1} segment. Cytoduction experiments showed that the splicing factor responsible for \textit{cox1}-I3β splicing in the \textit{S. paradoxus} strain CBS 432 and \textit{S. cariocanus} CBS 7994 mt genomes is coded by mtDNA. To find the particular gene we made a collection of petite mutants from a cybrid with \textit{S. paradoxus} CBS 432 mtDNA and crossed them with the non-adapted cybrid with mtDNA from \textit{S. paradoxus} CBS 7400. All petite genomes capable of complementing the glycerol growth defect were mapped to the \textit{cox1} gene. To shed more light on this puzzling difference, we sequenced \textit{cox1} (and entire mtDNAs) in all non-saccharomyces species as well in cybrids used in this study (Figure 11). The divergence in the \textit{cox1}-I3β sequence cannot by itself explain the differences in splicing efficiency among the different \textit{S. paradoxus} strains. Sequencing revealed only minor changes in the loop in P8 (insertion of ATAAATAA pos. 298 and (C→A) transition in pos. 328, out of the catalytic core of intron) between the form in \textit{S. paradoxus} CBS 432 (does not need adaptation) and two other \textit{S. paradoxus} strains (needing adaptation; Prochazka et al., 2012, Supplementary Material). The comparison of open reading frames, hypothetically coded for maturases, directed our attention toward the C terminus of the open reading frame inside \textit{cox1}-I5β, which is not fused to the upstream exon. It is nearly identical in mtDNAs of \textit{S. paradoxus} strain CBS 432 and \textit{S. cariocanus} CBS 7994, but interrupted by GC cluster in two strains that require an adaptation period. However, \textit{cox1}-I5β is not mobile and its splicing requires at least five different nuclei-coded splicing factors (Mrs1, Mss116, Pet54, and perhaps open reading frame coded by \textit{cob}-I3; Watts et al., 2011).

We tried to identify a fast evolving nuclear gene allowing the adaptation to “foreign” mtDNAs. A remarkable (tax reminiscent)
feature of the adaptation process was the loss of the ability to grow on glucose at elevated temperature. The phenotype was maintained even after mtDNA elimination, and was recessive as diploids from crosses with the \textit{S. cerevisiae} \(\rho^+\) strain were temperature resistant. Therefore, we attempted to find a gene from low copy and multicopy libraries by selecting for the ability to grow at 37°C, but we did not succeed, mainly due to high rate of reversion accompanied by the loss of the ability to grow well on glycerol.

When non-adapted cybrid with mtDNA \textit{S. paradoxus} CBS 7400 was transformed with libraries and glycerol growing colonies, selected \textit{MRS2}, \textit{MRS3}, \textit{MRS4} genes were identified as multicopy suppressors. All these genes were originally isolated as suppressors of a mutation in the first introns of \textit{cob} and \textit{cox1} genes in \textit{S. cerevisiae} impaired in splicing (Koll et al., 1987; Waldherr et al., 1993). All \textit{MRS}s code for mitochondrial transport proteins (reviewed by Arco and Satrústegui, 2005). \textit{MRS2} was identified as a Mg\(^{2+}\) carrier (Kolisek et al., 2003) and another two carriers, \textit{MRS3} and \textit{MRS4}, were shown to be involved in iron transport (Foury and Roganti, 2002; Mühlenhoff et al., 2003). Apparently, none of these genes is responsible for adaptation. Overexpression of the \textit{MRS3} and \textit{MRS4} causes temperature-dependent growth on glycerol but not on glucose, and the disruption of these genes affected neither the mitochondrial functions nor cellular viability (Wiesenberger et al., 1991). Strains with \textit{MRS2} disruption are unable to grow on non-fermentable substrates (Wiesenberger et al., 1992). None of the \textit{MRS} genes induce a temperature sensitive phenotype on glucose in non-adapted cybrids, but not in cells containing aboriginal \textit{S. cerevisiae} mtDNA. However, its overexpression does not trigger the temperature sensitive phenotype on glucose.

The third class is the \textit{S. cerevisiae} cybrid with mtDNA from \textit{S. bayanus} CBS 380, which is unable to re-establish growth on the non-fermentable carbon source even after 60 days on YPGE plates. This cybrid does not respire and glycerol growth can be restored only after mating with \textit{mit}+, which does not have a defect in the \textit{cox1} gene. Respiring xenomitochondrial cybrids with \textit{S. cerevisiae} mtDNA in \textit{S. bayanus} cannot be prepared either (Sulo et al., 2003). This establishes the nucleo-mitochondrial compatibility limit of \textit{S. cerevisiae} and other \textit{Saccharomyces} species between \textit{S. kudriavzevii} and \textit{S. bayanus} (Figure 12). The numbers concerning the divergence of particular \textit{Saccharomyces} species are still being developed. However, multiple calculations have estimated that \textit{S. paradoxus} and \textit{S. cerevisiae} diverged more than 7–10 million years ago and \textit{S. cerevisiae}, \textit{S. bayanus} more than 20 million years ago (www.genetics.wustl.edu/saccharomycesgenomes; Lin et al., 2006; Scannell et al., 2011). The \textit{S. paradoxus}/\textit{S. cerevisiae} rate of divergences compared with mammals (Dujon, 2006).

Figure 12 | Compatibility limit of \textit{S. cerevisiae} nuclear genome and mitochondrial genomes from related yeasts. Phylogenetic tree of \textit{Saccharomyces} species based on the comparison of (A) nuclear 26S rDNAs sequence and (B) mitochondrial \textit{cox2} gene. Branch lengths, based on nucleotide substitutions, are indicated by the bar. Bootstrap values of 1000 replications are marked by arrows at the branches points. Values less than 50% are not given. Schizosaccharomyces pombe was used as outgroup.
corresponds to the human–rods span and *S. cerevisiae/S. bayanus* to the human–birds distance. The limit confirms the transfer of *S. cerevisiae* mtDNA in the opposite direction. MtDNA containing all introns or intronless form is able to restore respiration in *S. paradoxus*, *S. kudriavzevii* and *S. mikatae* but not in *S. bayanus* to a level close to the original without adaptation period (unpublished). This emphasizes the unidirectional character of nucleo-mitochondrial incompatibilities and in particular the significant role of unusual *cox1*-I3β intron.

In conclusion, the interactions of *MRSl–cox1* considered as cytonuclear Dobzhansky–Muller pairs of speciation between *S. cerevisiae* and *S. paradoxus* are not strong enough to determine separation of species (Chou et al., 2010). Also, they do not play a dominant role in the separation between *S. cerevisiae* and the rest of the Saccharomyces species with the exception of species from the *S. bayanus–S. uvarum* clade (Figure 12). The compatibility is strain specific as the mitochondrial genomes can cooperate immediately and well-enough to support the utilization of non-fermentable substrate and sexual propagation. In the latter case, nuclear and mitochondrial genomes are capable of establishing collaborative communication after adaptation of one or several nuclear genes. Clear and strong cytonuclear incompatibility has been observed only between *S. cerevisiae* and *S. bayanus*, where additional Dobzhansky–Muller pairs have been reported (Chou et al., 2010).

Generally the post-zygotic barrier of speciation resulting in hybrid inviability and hybrid sterility has been attributed to a large number of genomic regions across a wide variety of species (for reviews Maheshwari and Barbash, 2011; Nosil and Schluter, 2011; Butlin et al., 2012; Mensch et al., 2013). In higher eukaryotes most of them involve chromosomal rearrangements and incompatibilities in nuclear Dobzhansky–Muller gene pairs (Maheshwari and Barbash, 2011). The yeasts with prevailing mito-nuclear incompatibilities are exception from this concept, although analogous mismatch have been found also in higher eukaryotes. MtDNA from chimpanzee, pigmy chimpanzee and gorilla but not from orangutan and less related primates is capable of establishing mitochondrial complex I deficiency, but can recover respiration with simultaneous phosphorylation in human cells devoid of mtDNA to essentially normal levels (Kenyon and Moraes, 1998). Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. *J. Biol. Chem.* 273, 14210–14217. doi: 10.1074/jbc.273.23.14210

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