The Mitochondrial Genome Impacts Respiration but Not Fermentation in Interspecific Saccharomyces Hybrids

Warren Albertin1,2*, Telma da Silva3*, Michel Rigoulet4,5, Benedicte Salin4,5, Isabelle Masneuf-Pomarede1,2, Dominique de Vienne6, Delphine Sicard6, Marina Bely1, Philippe Marullo1,7*

1 Univ. de Bordeaux, ISVV, EA 4577, Unité de recherche CEnologie, Villenave d’Ornon, France, 2 Bordeaux Sciences Agro, Gradignan, France, 3 INRA, UMR 0320/UMR 8120 Génétique Végétale, Gif-sur-Yvette, France, 4 CNRS, UMR 5095, Institute of Biochemistry and Genetics of the Cell, Bordeaux, France, 5 Univ. de Bordeaux, IBGC, UMR 5095, Bordeaux, France, 6 Univ Paris-Sud, UMR 0320/UMR 8120 Génétique Végétale, Gif-sur-Yvette, France, 7 BIOLAFFORT, Bordeaux, France

Abstract

In eukaryotes, mitochondrial DNA (mtDNA) has high rate of nucleotide substitution leading to different mitochondrial haplotypes called mtotypes. However, the impact of mitochondrial genetic variant on phenotypic variation has been poorly considered in microorganisms because mtDNA encodes very few genes compared to nuclear DNA, and also because mitochondrial inheritance is not uniparental. Here we propose original material to unravel mtotype impact on phenotype: we produced interspecific hybrids between S. cerevisiae and S. uvarum species, using fully homozygous diploid parental strains. For two different interspecific crosses involving different parental strains, we recovered 10 independent hybrids per cross, and allowed mtDNA fixation after around 80 generations. We developed PCR-based markers for the rapid discrimination of S. cerevisiae and S. uvarum mitochondrial DNA. For both crosses, we were able to isolate fully isogenic hybrids at the nuclear level, yet possessing either S. cerevisiae mtDNA (Sc-mtDNA) or S. uvarum mtDNA (Su-mtDNA). Under fermentative conditions, the mitotype has no phenotypic impact on fermentation kinetics and products, which was expected since mtDNA are not necessary for fermentative metabolism. Alternatively, under respiratory conditions, hybrids with Sc-mtDNA have higher population growth performance, associated with higher respiratory rate. Indeed, far from the hypothesis that mtDNA variation is neutral, our work shows that mitochondrial polymorphism can have a strong impact on fitness components and hence on the evolutionary fate of the yeast populations. We hypothesize that under fermentative conditions, hybrids may fix stochastically one or the other mt-DNA, while respiratory environments may increase the probability to fix Sc-mtDNA.

Introduction

Eukaryotes possess a cytoplasmic organelle called mitochondrion, either fully functional or vestigial [1,2]. Mitochondria are thought to originate from endosymbiosis between eukaryote’s ancestry and α-proteobacteria. This endosymbiotic event, first proposed by Wallin [3] and popularized by Sagan [4], may have arisen more than two billion years ago [5]. However, nowadays mitochondrial genomes contain far less genes than the genomes of α-proteobacteria [6]. Following endosymbiosis, most of the genes of the endosymbiote were either lost or transferred to the host cell genome during evolution [7]. While mitochondria are complex organelles requiring several hundred proteins to function properly, most of them (>99%) are now the product of nuclear genes. Mitochondrial genomes encode very few genes, between 3 and 96 genes in animals, plants, fungi and protozoa [7,8] and the proportion of genes encoded by mtDNA in Eukaryotes usually represents less than 0.5% of the total number of genes.

Mitochondrial gene content varies in a large extent among eukaryotes, with several lineage-specific variations in rates of gene loss. For example, 5 S rRNA is present only in land plants, some green algae, red algae, brown algae and protists [6], implying many independent and repeated losses of the 5 S tRNA gene across eukaryotic evolution. Identically, the number of tRNA genes encoded by mtDNA varies greatly across eukaryotes, ranging from none to around 30 tRNAs genes. In contrast, two major sets of mitochondrial genes are remarkably well conserved, those involved in respiration and in protein synthesis [6].

Unlike nuclear DNA (nuDNA), mtDNA has high rate of nucleotide substitution [9,10], so that several mitochondrial haplotypes (so-called mtotypes) coexist within species. The analysis of mitochondrial genetic diversity is widely used in population genetics to follow uniparental transmitted markers. However, the importance of mitochondrial genetic variation on phenotypic variation is scarcely considered, firstly because mtDNA encodes very few genes compared to nuDNA, and because mtDNA genetic variation has long been thought to be neutral [11,12]. In recent years, several studies revisited this longstanding view and showed that mtDNA variation might impact various...
phenotypic traits [13]. For example, in human, two mtDNA haplotypes were shown to be associated with human survival [14]. Other association studies showed that specific mtDNA mutations in humans are associated with oxygen consumption [15], athletic performance [16], sperm motility [17], Parkinson disease [18], adaptation to diet change and climate [19]. In other animals, cold acclimation was also shown to be associated with mtotypes in the greater white-toothed shrew, Crocidura muscula [20]. Mitochondrial polymorphism is associated with muscle composition in pig [21] or with resistance to insecticide in an arthropod pest (Tetanychus urticae) [22]. Nearly isogenic lines of Drosophila simulans, differing for mtDNA, showed important variations for fitness traits (longevity, activity, oxygen consumption, etc) [23–25]. In mice, ‘transmitochondrial hybrids’, resulting from the transfer of mitochondria to a mtDNA-less receptor cell line, varied for oxidative phosphorylation performances [26]. In plants and fungi also, cytoplasmic variants are related to fitness traits like in Silene vulgaris [27] or in the common button mushroom Agaricus bisporus [28].

However, most of these studies were performed at the population level or involved nearly isogenic lines. Indeed, it is very difficult to establish that mtDNA variants are actually population level or involved nearly isogenic lines. Indeed, it is possible very difficult to establish that mtDNA variants are actually related to fitness traits like in Agaricus bisporus or in Silene vulgaris variants are related to fitness traits like in Drosophila simulans, differing for mtDNA, showed important variations for fitness traits (longevity, activity, oxygen consumption, etc) [23–25]. In mice, ‘transmitochondrial hybrids’, resulting from the transfer of mitochondria to a mtDNA-less receptor cell line, varied for oxidative phosphorylation performances [26]. In plants and fungi also, cytoplasmic variants are related to fitness traits like in Silene vulgaris or in the common button mushroom Agaricus bisporus [28].

In this work we took advantage of the particular mitochondrial inheritance of the Saccharomyces species [37]. Saccharomyces yegetes result from the fusion of two parental cells, each having its own mitochondrial DNA. Thus, in the very first generations after hybridization, hybrids possess both parental mtDNA, which is called heteroplasmy [38]. This heteroplasmatic status is only transient and after a few generations (less than 20 divisions), homoplasmic cells harboring only one parental mtDNA are recovered [39]. In some cases, recombination between parental mtDNA may arise [40], yet only one recombined mtDNA is recovered after a few generations. The transition from heteroplasmy to homoplasmy can be stochastic [41,42] or non-stochastic [38,43]. Thus, it is theoretically possible to obtain fully isogenic hybrids resulting from the same cross, but harbouring one or the other of the two parental mtDNA.

In a previous work, Solier et al. [38] showed that interspecific hybrids between S. cerevisiae and S. warum may have increased respiratory ability when harbouring S. cerevisiae mtDNA compared to S. warum one. However, the synthetic interspecific hybrids tested differed regarding both mtDNA and nuDNA, so that it was difficult to assess whether differences in fermentative and respiratory performances were actually due to mtDNA by itself.

In this work, we produced interspecific hybrids between S. cerevisiae and S. warum species, using fully homoyzogous diploid parental strains. For two different interspecific crosses involving different parental strains, we recovered 10 independent hybrids per cross, and allowed mtDNA fixation after around 80 generations. For both crosses, we were able to isolate fully isogenic hybrids at the nuclear level, yet possessing either S. cerevisiae mtDNA (Sc-mtDNA) or S. warum mtDNA (Su-mtDNA). These hybrids were used to test the phenotypic impact of mitochondrial inheritance under respiratory conditions. In addition, even though it has long been suggested that mtDNA do not play any role in fermentation, indirect evidences suggested that actually they could [44]. Accordingly Sc-mtDNA and Su-mtDNA hybrids were also compared under fermentative conditions.

Materials and Methods

Yeast Strains and Culture Conditions

Eleven strains of Saccharomyces cerevisiae and four strains of S. warum were selected (Table 1). Monosporic clones were isolated from all these strains using a micromanipulator (Singer MSM Manual; Singer Instrument, Somerset, United Kingdom). All strains but Alcotec 24 and NRRL-Y-7327 were homoallelic (HO/ HO), so that the monosporic derivatives were fully homoyzogous diploid. For Alcotec 24 and NRRL-Y-7327 (ho/ho), the isolated haploid meiospore were diploidized via transient expression of the HO endonuclease (see Albertin et al., 2009 [45]). These fully homoyzogous diploid strains, called W1–W2, D1–D2, B1–B2, E1–E5 for S. cerevisiae and U1–U4 for S. warum were used for subsequent analysis of the genetic diversity of mitochondrial DNA and for interspecific hybrid construction.

All strains were usually grown at 24°C in YPD medium containing 1% yeast extract (Difco Laboratories, Detroit, MI), 1% Bacto peptone (Difco), and 6% glucose, supplemented or not with 2% agar. When necessary, antibiotic concentration was as followed: 100 μg/mL for G418 (Sigma, France), 300 μg/mL for hygromycin B (Sigma, France), and 100 μg/mL for nourseothricin (Sigma, France).

For a quick assessment of respiratory-ability, cells were plated on YPGly medium, containing glycerol as unique source of carbon: 1% yeast extract (w/v, Difco Laboratories, Detroit, MI), 1% Bacto peptone (w/v, Difco), and 6% glucose, supplemented or not with 2% agar. When necessary, antibiotic concentration was as followed: 100 μg/mL for G418 (Sigma, France), 300 μg/mL for hygromycin B (Sigma, France), and 100 μg/mL for nourseothricin (Sigma, France).

Mitochondrial DNA Sequence

Genomic DNA extraction were performed as described by Albertin et al [46] or by using FTA® CloneSaver™ Card (Whatman®BioScience, USA). Three mitochondrial loci, COX3, COX2 and ATP6, were sequenced in 11 S. cerevisiae and 4 S. warum fully homoyzogous strains. An additional locus VAR1 was sequenced only for S. cerevisiae strains. Both strands of PCR products were sequenced using Sanger method (GATC biotech, Germany). The sequences were aligned with ClustalW using the BioEDIT program [47]. Aligned fragments were deposited in EMBL (accession numbers HF951713–HF951770). The genetic distance between sequences (number of differences per base) was estimated using MEGA 5 software [48]. Phylogenetic trees were build using the Neighbor-Joining method [49] with bootstrap implementation (500 iterations).

Mitochondrial Genotyping

The three loci COX2, COX3 and ATP6 were used to design degenerated primers able to amplify in a single PCR reaction the S. cerevisiae and S. warum alleles. The COX2 primers used were previously described by Belloch et al. [50] and required the digestion of PCR fragment by the endonuclease SfiI. The ATP6 and COX3 primers allow differentiating S. warum and S. cerevisiae by
### Table 1. Characteristics of *Saccharomyces cerevisiae* and *S. uvarum* strains used.

| Species       | Strain       | Genotype | Ploidy | Collection/ supplier* | Origin                          | Reference                        |
|---------------|--------------|----------|--------|-----------------------|---------------------------------|----------------------------------|
| *S. cerevisiae* | YSP128       | HO/HO    | diploid| SGRP                  | Forest Oak exudate, Pennsylvania, USA | Liti et al., 2009 [60]          |
| *S. cerevisiae* | UWOPS83-787.3 | HO/HO    | diploid| SGRP                  | Fruit Opuntia stricta, Bahamas   | Liti et al., 2009 [60]          |
| *S. cerevisiae* | Alcotec 24   | ho/ho    | diploid| Hambleton Bard        | Distillery, UK                   | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | CLIB-294     | HO/HO    | diploid| CIRM-Levures           | Cognac, France                   | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | CLIB-328     | HO/HO    | diploid| CIRM-Levures           | Enology, UK                      | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | CLIB-382     | HO/HO    | diploid| CIRM-Levures           | Brewery, Japan                    | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | VL1          | HO/HO    | diploid| Laftort Ënologie       | Enology, Bordeaux, France         | Marullo et al., 2006 [88]        |
| *S. cerevisiae* | F10          | HO/HO    | diploid| Laftort Ënologie       | Enology, Bordeaux, France         | Marullo et al., 2009 [89]        |
| *S. cerevisiae* | VL3c         | HO/HO    | diploid| Laftort Ënologie       | Enology, Bordeaux, France         | Marullo et al., 2004 [90]        |
| *S. cerevisiae* | BO213        | HO/HO    | diploid| Laftort Ënologie       | Enology, Bordeaux, France         | Marullo et al., 2006 [88]        |
| *S. cerevisiae* | NRRL-Y-7327  | ho/ho    | diploid| NRRL                  | Brewery, Tibet                   | Albertin et al., 2009 [45]      |
| *S. cerevisiae* | PM12         | HO/HO    | diploid| ISVV                   | Grape must, Jurançon, France      | Naumov et al., 2000 [91]         |
| *S. cerevisiae* | PJF3         | HO/HO    | diploid| ISVV                   | Grape must, Sancerre, France      | Naumov et al., 2000 [91]         |
| *S. cerevisiae* | Br6.2        | HO/HO    | diploid| ADRIA Normandie       | Cider fermentation, Normandie, France | Demuyter et al., 2004 [92]  |
| *S. cerevisiae* | RC4-15       | HO/HO    | diploid| ISVV                   | Grape must, Alsace, France        | Demuyter et al., 2004 [92]      |
| *S. cerevisiae* | W1           | monosporic clone of YSP128, HO/HO | diploid| ISVV                  |                                | Blein et al., 2013 [62]          |
| *S. cerevisiae* | W2           | monosporic clone of UWOPS83-787.3, HO/HO | diploid| ISVV              |                                | this work                        |
| *S. cerevisiae* | D2           | monosporic clone of Alcotec 24, ho/ho | diploid| ISVV                |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | D1           | monosporic clone of CLIB-294, HO/HO | diploid| ISVV                |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | E1           | monosporic clone of CLIB-328, HO/HO | diploid| ISVV                |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | B1           | monosporic clone of CLIB-382, HO/HO | diploid| ISVV               |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | E3           | monosporic clone of VL1, HO/HO | diploid| ISVV                |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | E4           | monosporic clone of F10, HO/HO | diploid| ISVV                |                                | Albertin et al., 2011 [46]      |
| *S. cerevisiae* | E5           | monosporic clone of VL3c, HO/HO | diploid| ISVV                |                                | Blein et al., 2013 [62]          |
| *S. cerevisiae* | E2           | monosporic clone of SB, HO/HO | diploid| ISVV                |                                | Marullo et al., 2009 [89]        |
| *S. cerevisiae* | B2           | monosporic clone of NRRL-Y-7327, HO/HO | diploid| ISVV               |                                | Blein et al., 2013 [62]          |
| *S. uvarum*    | U1           | monosporic clone of PM12, HO/HO | diploid| ISVV                |                                | Blein et al., 2013 [62]          |
| *S. uvarum*    | U2           | monosporic clone of PJF3, HO/HO | diploid| ISVV                |                                | Blein et al., 2013 [62]          |
| *S. uvarum*    | U3           | monosporic clone of Br6.2, HO/HO | diploid| ISVV               |                                | Blein et al., 2013 [62]          |
| *S. uvarum*    | U4           | monosporic clone of RC4-15, HO/HO | diploid| ISVV               |                                | this work                        |
| *S. cerevisiae* | D2-3A-HYG    | ho::hygR, MATx | haploid| ISVV               |                                | this work                        |
| *S. cerevisiae* | W1-NAT-1B    | ho::natR, MATa | haploid| ISVV               |                                | this work                        |
| *S. uvarum*    | U2-KAN-3B    | Suho::kanR, MATx | haploid| ISVV              |                                | this work                        |
| *S. uvarum*    | U3-KAN-3A    | Suho::kanR, MATa | haploid| ISVV             |                                | this work                        |

*Laffort Ënologie: http://www.laffort.com; CIRM-Levures (Centre International de Ressources Microbiennes): http://www.inra.fr/internet/Produits/cirmlevures; NRRL (Northern Regional Research Laboratory, now Agricultural Research Service Culture Collection): http://nrrl.ncias.usda.gov; Hambleton Bard: http://www.hambletonbard.com; ISVV (Institut Scientifique de la Vigne et du Vin): http://www.oenologie.u-bordeaux2.fr/; ADRIA Normandie: http://www.adria-normandie.com; SGRP (Saccharomyces Genome Resequencing Project): http://www.sanger.ac.uk/research/projects/genomeinformatics/sgrp.html.

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the PCR product length (Table S1). An additional locus, VARI can be used to discriminate Saccharomyces cerevisiae strains by an RFLP approach (Table S1). The PCR reactions were carried out with 2–6 ng of genomic DNA extract as template, 1X Taq-&GO master mix for PCR (Qiogene), in 20 µL final volume. PCR fragment sizes were analyzed by capillary electrophoresis with a multi NA apparatus (Shimatzu, Germany) using the 1000 pb gel kit.

Hybrid Construction

In order to produce interspecific hybrids, two diploid parental strains per species (W1 and D2 for S. cerevisiae, U2 and U3 for S. warum) were transformed with a cassette containing the HO allele disrupted by a gene resistance to either G418 or kanR, hygromycin B or nourseothricin. For S. cerevisiae strains, the ho·kanR, ho·hygR and ho·natR cassettes were respectively amplified by PCR using the following primers p25: TGGTTTACGAAATGTCCAC, p26: AAATCGAGACGCATCTGTG and the genomic DNA of the strains BY4741 (Euroscarf, Frankfurdt, Germany), YG1 and YG13 (kindly given by Professor Richard Gardner, Auckland, New Zealand). For S. warum strains, the Subho·kanR cassette containing the KanMX4 coding sequence (1506 bp) flanked on 5’ and 3’ by 500 bp flanking- sequence of S. warum HO gene was synthesized by Genscript and cloned in the pUC57 vector. This cassette was then amplified by PCR using the primers p599 TACCCAGGAAAAAGTATGTAATG and p600 CTTATCCTGAGCGTATGGCCG. For all ho-disruption cassettes amplification, the PCR mix contained 100–600 ng of DNA template, 0.1 mM of each primer, 1X Taq-&GO master mix for PCR (Qiogene), in 100 µL final volume. The PCR reaction was as followed: 3 minutes at 94°C, followed by 35 cycles – 30 seconds at 94°C, 30 seconds at 54°C or 55°C (for S. cerevisiae and S. warum cassettes respectively), 3 minutes at 72°C – and a final elongation step of 5 minutes at 72°C.

Strains were transformed using the lithium acetate protocol described by Gietz and Schiestl [51] for all S. cerevisiae strains and for U4, and alternatively using the frozen yeast TRAFO protocol [52] for U1, U2 and U3. After transformation, monosporic clones were isolated, and the mating-type (MATa or MATa) of antibiotic-resistant clones was determined using tests of well-known mating-type. Strain transformation allowed (i) conversion to heterothallism for the homothallic strains (all but B2 and D2, see Table 1) and (ii) antibiotic resistance allowing easy hybrid production.

For DU23 hybrids, the parental strains D2-3A-HYG (MATa) and U3-KAN-3A (MATa) were pulled in contact two to four hours in YPD medium at room temperature, and then plated on YPD-agar with G418 and hygromycin B. The same procedure was applied for WU12 hybrids whose parental strains were W1-NAT-1B (MATa) and U2-KAN-3B (MATa) and were thus selected on YPD-agar added with G418 and nourseothricin. Ten independent hybrids per cross were recovered. Recurrent cultures on YPD-agar (24°C), each from one colony, which corresponded to ~80 generations, were made in order to allow mitochondrial fixation (homoplasy) and to assess hybrids chromosomal stability through multiple generations.

Hybrid Characterization

Karyotype analysis of the hybrids and their corresponding progenitors was carried out using pulse-field gel electrophoresis (PFGE). Briefly, chromosomal DNA was prepared from overnight cultures in agarose plugs as described by Bellis et al. [53]. Chromosomes were separated with a CHEF DRRII apparatus (Bio-Rad, Richmond, CA, USA) on a 1% agarose gel (Qiogene, Carlsbad, CA, USA) and using TBE as running buffer. Electrophoresis was carried out at 200 V and 10°C for 16 h with a switching time of 60 ms, and then for 10 h with a switching time of 105 ms. DNA was bound by bromide ethidium staining (30 minutes).

In addition to PFGE, hybrids were characterized by PCR ribotyping (5.8S–ITS rDNA amplification followed by HaeIII restriction) allowing discrimination between S. cerevisiae and S. warum strains [38,34,55].

Fermentation Assays

White grape must was obtained from Sauvignon grapes, harvested in vineyards in Bordeaux area (2009 vintage). Tartaric acid precipitation was stabilized and turbidity was adjusted to 100 NTU (Nephelometric Turbidity Unit) before long storage at 20°C. Sugar concentration was 188 g L⁻¹, and the indigenous yeast population, estimated by YPD-plate counting after must thawing, was low, i.e. less than 20 CFU (colony-forming unit) per mL.

Pre-cultures were run in half-diluted must filtered through a 0.45 µm nitrate-cellulose membrane, during 24 h, at 24°C with orbital agitation (150 rpm). Population size was measured using a flow cytometer (see below). Sauvignon must was inoculated at 10⁶ viable cells per mL. Fermentation triplicates were run in closed 125 mL glass-reactors, locked to maintain anaerobiosis, with permanent stirring (300 rpm) at 18°C. The CO₂ released was allowed by a needle and was determined by measurement of glass-reactor weight loss regularly and the CO₂max was calculated as the maximal CO₂ released in g L⁻¹. The fermentation kinetics data were fitted with logistic model allowing the calculation of several kinetics parameters: lag phase time (h) was the time between inoculation and the beginning of CO₂ release. AF time (h) was the time to complete alcoholic fermentation (without lag-phase). Vmax was the maximal rate of CO₂ release in g L⁻¹ h⁻¹.

At the end of the alcoholic fermentation, ethanol concentration (percent volume) was determined by infrared reflectance (Infra-Analyzer 450; Technicon, Plains, France), acetic acid production (g L⁻¹) was measured by colorimetry (H460) in continuous flux (Sanimat, Montauban, France) and both residual D-glucose and D-fructose (g L⁻¹) were quantified using an enzymatic method (Kit D-glucose/D fructose Boehringer, Germany) in the supernatant.

External glycerol (g L⁻¹) was assayed by the enzymatic method (Boehringer kits 10 148 270 035, R-Biopharm, Darmstadt, Germany).

Cell Growth Conditions for Respiratory Assays

Respiratory growth was assessed on YPEG medium containing 1% yeast extract (w/v, Difco Laboratories, Detroit, MI), 1% Bacto peptone (w/v, Difco, Detroit, MI), 3% ethanol (v/v) and 3% glycerol (v/v). Pre-cultures were run in half-diluted YPEG medium during 24 h, at 28°C with orbital agitation (150 rpm). Population size was measured using flow cytometry (see below) to inoculate YPEG at 10⁶ viable cells per mL. Triplicates were run in 200 mL Erlenmeyers containing 50 mL YPEG medium, with high permanent stirring (900 rpm) to favour oxygenation at 28°C.

Population Dynamics Using Flow Cytometry

Regularly, cells were sampled and population size was estimated using a flow cytometer (Quanta SC MPL, Beckman Coulter, France), equipped with a 488 nm laser and a 670 nm long-pass filter, at 22 mW. Samples were diluted in McIlvaine buffer pH 4.0 (0.1 M citric acid, 0.2 M sodium phosphate dibasic) added with propidium iodide (0.3% v/v) in order to stain dead cells (red fluorescence measure in FL3 channel). The experimental points were fitted with a logistic model [46] that allowed estimation of the
carrying capacity (maximum population size, \(K\), cells per mL) and the intrinsic growth rate \(r\) (number of divisions per hour).

**Oxygen Consumption Assays**

WU12-8 (Sc-\(mt\)DNA) and WU12-1 (Su-\(mt\)DNA) were grown aerobically in YPEG liquid medium, at 28°C. During exponential phase, the oxygen consumption was measured polarographically at 28°C using a Clark oxygen electrode in a 1-mL thermostatically controlled chamber. Distinct respiratory rates were considered: spontaneous respiratory rate \(J_{O_2}\text{spontaneous}\), which is oxygen uptake during growth conditions, uncoupled respiratory rate \(J_{O_2\text{uncoupled}}\), which is measured in the presence of 1 \(\mu\)M of the protonophoric uncoupler CCCP (carbonyl cyanide m-chlorophenylhydrazone, Sigma, France) and is an indication of the maximal respiratory rate achieved by the cells [56], and finally none-phosphorating respiratory rate \(J_{O_2\text{basal}}\) which is the residual respiratory rate measured when ATP synthase is inhibited in presence of 200 \(\mu\)M of TET (Tri Ethyl Tin chloride, Alfa Aesar, USA). The ATP respiration due to ATPase activity was estimated \(J_{O_2\text{ATP}}\) and the percentage of spontaneous respiration due to ATPase activity was estimated \(J_{O_2\text{ATP}/J_{O_2\text{basal}}}\).

All respiratory rates were determined from the slope of a plot of \(O_2\) concentration versus time and were expressed as nmol \(O_2/\min/10^6\) cells, population size being measured by flow cytometry. Four measures of all three respiratory rates were performed during exponential growth, and the experiment was performed in duplicate.

**Cytochrome Content Determination**

The cellular content of \(c+1\), \(b\), and \(a+3\) hemes was calculated as described by Dejean et al. [57], taking into account the respective molar extinction coefficient values and the reduced minus oxidized spectra recorded using a dual beam spectrophotometer (Aminco DW2000). Two to four measures were made, and cytochrome content was expressed in pmol/mg dry weight of cells.

**Electronic Microscopy**

Yeast pellets (after YPEG overnight growth) were placed on the surface of a copper EM grid (400 mesh) that had been coated with formvar. Each grid was very quickly submerged in liquid propane pre-cooled and held at \(-180^\circ\text{C}\) by liquid nitrogen. The loops were then transferred in a pre-cooled solution of 4% osmium tetroxide in dry acetone in a 1.8 ml polypropylene vial at \(-82^\circ\text{C}\) for 72 h (substitution), warmed gradually to room temperature, followed by three washes in dry acetone. Specimens were stained for 1 h in 1% uranyl acetate in acetone at 4°C, blackroom (epoxy resin Fluka). Ultrathin sections were contrasted with lead citrate. Specimens were observed with a HITACHII 7650 (80 kV) electron microscope (PIE, BIC, Bordeaux Segalen University).

**Statistical Analysis**

Within each cross (WU12 and DU23), the variation of each trait was investigated using the lm function (R program), through the following model of ANOVA:

\[
Z = \mu + \text{strain}_i + e_i
\]

where \(Z\) is the variable, \(\text{strain}\) is the strain effect (\(i = 1, 2, 3, 4\)) and \(e\) is the residual error. Within each cross, the four strains corresponded to two independent strains with Sc-\(mt\)DNA and two independent strains with Su-\(mt\)DNA. Since several traits were tested, \(P\) values were adjusted for multiple testing using Benjamini-Hochberg methods by means of R’s language, version 2.14.1 [58]. For each variable, the homogeneity of the variance was assessed using a Levene test by means of R’s \texttt{car} package version 2.14.1 [58], as well as the normality of residual distribution using a Shapiro test [59]. Duncan’s multiple comparison was used to determine which means differ significantly (Duncan’s multiple comparison, \(p<0.05\)).

**In silico Competition between Mitotypes**

Modeling population growth was made using the kinetics parameters calculated under respiratory conditions (YPEG medium) using a logistic model: \(K = 3.63.10^{10}\) cells per mL for both WU12 Sc-\(mt\)DNA and Su-\(mt\)DNA, \(r = 0.222\) and 0.196 division per hour for WU12 Sc-\(mt\)DNA and Su-\(mt\)DNA respectively; \(K= 3.29.10^{10}\) cells per mL for both DU23 Sc-\(mt\)DNA and Su-\(mt\)DNA, \(r = 0.207\) and 0.176 division per hour for DU23 Sc-\(mt\)DNA and Su-\(mt\)DNA respectively. The initial mixed population was of \(10^6\) cells per mL (ratio 1:1 Sc-\(mt\)DNA:Su-\(mt\)DNA). When the maximal population size was reached \((K)\), a new in silico culture was inoculated at \(10^6\) cells per mL, using the ratio of mitotypes (Sc-\(mt\)DNA:Su-\(mt\)DNA) calculated at the end of the preceding culture.

**Results**

**Mitochondrial Sequence Analysis in \(S. cerevisiae\) and \(S. uvarum\)**

In order to develop polymorphic mitochondrial markers for both \(S. cerevisiae\) and \(S. uvarum\) species, we sequenced three mitochondrial genes (COX3, COX2 and ATP6) for 11 \(S. cerevisiae\) and 4 \(S. uvarum\) strains. An additional loci \(VAR1\) was sequenced only for \(S. cerevisiae\) strains. To maximize the chance to find polymorphism, intergenic segments were amplified from flanking coding regions. This dataset allows a first study of the intra-specific variability of \(mt\)DNA within natural populations (Table 2). For \(S. cerevisiae\), sequence alignments of COX2, ATP6, COX3, and \(VAR1\) were performed for 12 strains, including the reference strain S288C. Depending on the gene, we identified 5 to 11 allelic forms. The genetic polymorphism varied greatly depending on the locus and the strain with an average of 2.53% nucleotide difference within the 12 strains. The COX2 and \(VAR1\) coding sequences display low polymorphism (0.34% and 0.43% nucleotide difference). By contrast, the promoters of ATP6 and COX3 promoter harbored more nucleotide polymorphism between strains (9.75% and 0.66% nucleotide difference, respectively). The promoter region of ATP6 was found to be particularly polymorphic due to the insertion of two CG clusters at different position defining two groups of strains. A multi-locus analysis was carried out concatenating these sequences (2650 positions). Wine yeasts were grouped together as illustrated by the phylogenetic tree presented in Figure 1, which is congruent with previous work studying nuclear DNA polymorphism [45,59,60].

For \(S. eubayanus\) \(uvarum\), there is no published mitochondrial genome. So we used \(S. pastorianus\) \(mt\)DNA genome as reference: \(S. pastorianus\) is an allotetraploid whose progenitors are \(S. cerevisiae\) and \(S. eubayanus\), a newly-described species phylogenetically closely to \(S. uvarum\). \(S. pastorianus\) inherited the mitochondrial DNA from \(S. eubayanus\) [61]. Regarding the three loci analyzed (COX2, COX3 and ATP6), the \(S. eubayanus\) \(mt\)DNA sequence is divergent from the four \(S. uvarum\) sequences with an average of 8.8% nucleotide difference for 1454 positions, while within \(S. uvarum\) few allelic variations were detected (0.30% nucleotide difference). Such a low genetic variability within \(S. uvarum\) in comparison to \(S. cerevisiae\) is
consistent with the results of a recent multilocus genotyping experiment carried out on six nuclear genes [62].

Development of Co-dominant Mitochondrial Markers for *S. cerevisiae* and *S. uvarum* Species

To have a readily and economic mtDNA genotyping, co-dominant mitochondrial markers were developed using either variation in length PCR-amplicon (PCR-LP) or PCR followed by RFLP. Although numerous nucleotide polymorphisms were found by sequencing, a relative few number of restriction sites were observed. For inter-specific discrimination, three markers were developed (*COX3*, *COX2* and *ATP6*) that allowed a clear discrimination between *S. cerevisiae* and *S. uvarum* (Figure 2). In addition, *ATP6* and *VAR1* loci displayed intra-specific polymorphism within *S. cerevisiae* species when the PCR product was digested with *Bpi* and *Btg* respectively. When combined together, those loci allowed differentiating five of the 11 mtDNA of the *S. cerevisiae* strains analyzed (Figure 2). By contrast, the very low polymorphism of *S. uvarum* species prevents the use of these mtDNA markers to discriminate *S. uvarum* strains (Table S1).

Interspecific Hybrid Construction and Characterization

Interspecific hybridization between *S. cerevisiae* and *S. uvarum* was performed, allowing us to get the hybrids DU23 (D2 × U3) and WU12 (W1 × U2). For each cross, ten independent hybrids were isolated and confirmed by amplification of the rDNA NTS2 region followed by *Hae* III restriction [63]. Recurrent cultures were then made, corresponding to 80 generations. Pulse-field gel electrophoreses were run to determine whether the hybrids actually possessed both parental chromosome sets. All 20 hybrids displayed additive karyotype, except DU23-2 (Figure 3) that presented large chromosomal rearrangements with additional and missing parental chromosome bands. This result indicated that inter-specific hybridization was relatively stable at the chromosomal level, even after 80 generations.

Mitochondrial inheritance was then assessed for these 20 interspecific hybrids to determine whether the different hybrids

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**Figure 1. Evolutionary relationships of *Saccharomyces cerevisiae* strains for mtDNA.** The phylogenetic tree was inferred by using the Maximum Likelihood method based on the Tamura-Nei model with bootstrapping (500 iterations). Branches corresponding to partitions reproduced in less than 80% bootstrap replicates are collapsed. The analysis involved 12 nucleotide sequences representing the concatenation of 4 mitochondrial loci (*COX2*, *COX3*, *VAR1* and *ATP6*). All positions containing gaps and missing data were eliminated. There were a total of 2719 positions in the final dataset. Evolutionary analyses were conducted in MEGAS. Label describes the origin of the strains: natural isolate, distillery, brewing, wine.

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**Table 2. Genetic diversity of *COX2*, *COX3*, *ATP6* and *VAR1* mtDNA loci.**

| Locus | Species (# strains) | Alignment size | Alleles number | Nucleotide difference range | Description | EMBL access |
|-------|---------------------|----------------|----------------|-----------------------------|-------------|-------------|
| COX2  | *S. cerevisiae* (12) | 527            | 5              | 0–4                        | *COX2* coding sequence | HF951745-48 |
|       | *S. uvarum* (4)     | 561            | 2              | 0                          |             | HF951749-60 |
| COX3  | *S. cerevisiae* (12)| 630–749       | 7              | 0–78                       | *COX3* promoter | HF951734-44 |
|       | *S. uvarum* (4)     | 704–507       | 3              | 0–6                        |             | HF951730-33 |
| ATP6  | *S. cerevisiae* (12)| 692–743       | 11             | 12–366                     | *ATP6* promoter | HF951719-29 |
|       | *S. uvarum* (4)     | 450–480       | 4              | 0–7                        |             | HF951715-18 |
| VAR1  | *S. cerevisiae* (12)| 971–1068      | 7              | 0–145                      | *VAR1* coding sequence | HF951760-70 |
|       | *S. uvarum*         | ND            | ND             | ND                         |             |             |

*a For *S. cerevisiae*, 12 sequences (11 strains + reference strain) were analyzed.

*b For *S. uvarum*, 4 sequences were analyzed, the sequence of the strain PM12 was used as reference.

*c Number of base differences per sequence respect to the reference. Results are based on the pairwise analysis conducted in MEGAS; all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

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had recovered Sc-mtDNA or Su-mtDNA. The mtDNA was genotyped after 20 and 80 generations (Table S2). Depending on the interspecific cross, the results varied: after 20 generations, only one case of heteroplasmy was detected among 10 independent WU12 hybrids, and after 80 generations all 10 hybrids had fixed either Sc-mtDNA (7/10 hybrids) or Su-mtDNA (3/10 hybrids). All these interspecific hybrids were able to grow on YPGly petri plate, containing glycerol as carbon source, indicating efficient respiration metabolism. By contrast, for DU23 background, two heteroplasmic hybrid strains were observed after 20 generations, as well as three hybrids with mtDNA recombination, most of them being respiratory-defective (unable to grow on YPGly petri plate). After 80 generations, four inter-specific hybrids displayed partial or complete mtDNA loss, associated with inability to grow on YPGly petri plate. Two hybrids with mtDNA recombination were observed, of which only one was able to respire. The four remaining inter-specific hybrids were homoplasmic, two of them with Sc-mtDNA, and two with Su-mtDNA.

Figure 2. Molecular markers for typing intra and interspecific variability of mtDNA in *S. cerevisiae* and *S. uvarum* species. Three interspecific markers (*S. cerevisiae* vs. *S. uvarum*) and two intra *S. cerevisiae* markers were developed using PCR and enzymatic restriction. The interspecific markers *ATP6* and *COX3* allowed the rapid identification of mitotypes by length polymorphism after PCR. The *COX2* marker required the digestion of PCR fragments by the enzyme SfcI to discriminate the two species mitotypes. For the identification of mtDNA within *S. cerevisiae* strains the ATP6 and VAR1 PCR fragments were digested with the restriction enzymes BpfI and BgtI, respectively. Combining both markers, five mitotypes could be identified. doi:10.1371/journal.pone.0075121.g002
Hybrids with Sc-mtDNA have Higher Growth Performance Under Respiratory Conditions

The possibility to obtain readily numerous inter-specific hybrids by antibiotic selection and the development of molecular test for assessing mitochondrial inheritance pave the way to investigate the phenotypic impact of mitochondrial inheritance in an isogenic context. This unique genetic material allows evaluating the impact of natural genetic variations of mtDNA on the fitness of inter-specific hybrids. For each interspecific cross (WU12 and DU23), we chose two independent homoplasmic hybrids with either Sc-mtDNA (WU12-8, WU12-9, DU23-1 and DU23-9) or Su-mtDNA (WU12-1, WU12-2, DU23-3 and DU23-4). These hybrids are thus fully identical at the nuclear level and differ only for mitochondrial DNA, allowing reliable study of the impact of mtDNA inheritance alone on phenotype. As the foremost function of mitochondria in yeast is glucose oxidation through cellular respiration, we first analyzed cell growth under respiratory conditions. The interspecific hybrids were grown in YPEG medium associated with strong permanent stirring and population was followed by flow cytometry analysis. For both crosses, interspecific hybrids having Su-mtDNA (WU12-1, WU12-2, DU23-3 and DU23-4) had apparent lower population size until the carrying capacity (maximal population size) was reached (Figure 4). Growth kinetics were fitted on logistic function to determine the lag phase time, the maximal population size, and the intrinsic growth rate. Variance analysis (ANOVA) revealed that interspecific hybrids reached similar maximal population size within each cross, indicating that mtDNA inheritance had no impact on final carrying capacity in interspecific hybrids (Table 3). By contrast, lag phase time and intrinsic growth rate were strongly affected; for WU12 cross, hybrids with Su-mtDNA had increase lag phase time (15.4 and 16.1 hours for WU12-1 and WU12-2 respectively) than hybrids with Sc-mtDNA (13.2 and 13.8 hours for WU12-8 and WU12-9, respectively). In addition, Su-mtDNA hybrids showed lower intrinsic growth rate than hybrids with Sc-mtDNA (0.201 and 0.191 division per hour for WU12-1 and WU12-2 respectively, compared to 0.224 and 0.221 division per hour for WU12-8 and WU12-9, respectively). The same features were observed for DU23 cross, with Su-mtDNA hybrids having higher lag phase time (around 16.6 hours) and lower growth rate (around 0.175) compared to Sc-mtDNA hybrids (14 hours of lag phase and 0.207 division per hour). In both interspecific crosses, Su-mtDNA inheritance was associated with delayed and slower growth compared to Sc-mtDNA.

Hybrids having Sc-mtDNA have Higher Respiratory Rate

To go further, the respiratory ability of WU12 hybrids was investigated. Four different respiratory rates were measured: the spontaneous respiratory rate (\(J_0s\), which is oxygen uptake under non-limiting growth conditions), the uncoupled respiratory rate, a proxy for the maximal respiratory rate (\(J_0max\) achieved by the cells [36]), the none phosphorylating respiratory rate (basal \(J0\)) which is the residual respiratory rate measured when ATPsynthase is inhibited and finally the ATPase respiratory rate coupled to ATP synthesis (\(J0_{ATP}\)), which is the respiratory rate due to ATP synthesis functioning. For all respiratory rates, the hybrid having Sc-mtDNA (WU12-8) showed higher respiratory ability (Table 3), with a similar increase of 60% compared to Su-mtDNA (WU12-1). By contrast, the proportion of respiratory rate associated with ATPase functioning was identical (73–76%) in both hybrids. Such a large increase in respiratory rates could be due either to differences in mitochondria number and/or volume, or to variation in intrinsic mitochondrial respiratory abilities. To test these hypotheses, we first run electron microscopy of both hybrids (Figure S1). There was no evident difference in the number of mitochondria, their volume and the number of observed cristae, indicating that both hybrids displayed similar qualitative and quantitative mitochondrial content, independent of mtDNA heredity. We then measured the cellular content in cytochromes \(a+c+c1\) and \(a+a3\). WU12-8 Sc-mtDNA showed significant lower content in cytochrome \(a+c+c1\) cytochromes, as well as significant higher content in cytochrome \(a+a3\), in comparison with WU12-1 Su-mtDNA. Interestingly, for cytochrome \(a+a3\), a similar trend was observed for DU23 hybrids: DU23-1 Sc-mtDNA harboured higher yet not significant \(a+a3\) cytochrome content (10.6 pmol/mg dry weight) compared to DU23-4 Su-mtDNA (7.4 pmol/mg dry weight). The cytochrome content of the parental strains revealed that both S. cerevisiae parental strains (W1 and D2) had significant higher content in \(a+a3\) cytochromes (10.0 pmol/mg dry weight) compared to S. uvarum strains U2 and U3 (6.2 pmol/mg dry weight), suggesting that variation in \(a+a3\) cytochrome content might be related to the mitotype. It has been shown in yeast that the respiratory rate is mainly controlled by cytochrome oxidase activity [64] and that during growth on non-fermentable carbon source, cell respiratory rate is directly proportional to cytochromes \(a+a3\) content [65]. Thus, the fact that WU12-8 Sc-mtDNA harboured higher \(a+a3\) cytochrome content than WU12-8 Su-mtDNA explains the difference observed in respiratory rate between both hybrids during growth.

Mitotype has no Phenotypic Impact on Fermentation Kinetics and Products

For a long time, mitochondrion was thought to be useless under fermentative conditions, mainly because cells with defective respiration were able to ferment normally [66]. In addition, many genes encoding mitochondrial proteins are repressed under fermentative conditions associating high glucose content and anaerobia [67,68]. However, several authors suggested that mitochondria may be critical for yeast fermentative performance [44,69]. Therefore we assessed the possible effect of mitochondrial genotype under fermentative conditions. Alcoholic fermentations were run in grape must, and parameters related to fermentation kinetics (Figure 5) were measured (lag phase time, \(\Delta F\) time, \(CO2max\), \(Vmax\)). In addition, at the end of the fermentation, the main products (ethanol, acetic acid, glycerol) were measured, as well as the residual sugar (Table 4). Within each cross, all four strains,
Mitotype Impacts Respiration but Not Fermentation

### Table 3. Results of the ANOVAs: F values and Mean values for respiration parameters.

| Parameters | WU12 interspecific cross | DU23 interspecific cross |
|------------|--------------------------|--------------------------|
|            | ANOVA | Mean value +/- SD (Duncan’s class) | ANOVA | Mean value +/- SD (Duncan’s class) |
|            | Fvalue | df | WU12-1 Su | WU12-2 Su | WU12-8 Sc | WU12-9 Sc | Fvalue | df | DU23-1 Sc | DU23-2 Sc | DU23-4 Sc | DU23-9 Sc |
| K          | 3.34   | 3  | 3.96e+08 +/- 1.9e+07 | 3.91e+08 +/- 2.8e+07 | 3.51e+08 +/- 1.4e+07 | 3.75e+08 +/- 1.2e+07 | 1.92    | 3    | 3.44e+08 +/- 5e+06 | 3.49e+08 +/- 2.5e+07 | 3.16e+08 +/- 2.6e+07 | 3.13e+08 +/- 2.9e+07 |
| R          | 9.38** | 3  | 0.201e-06 +/- 0.006(a) | 0.191 +/- 0.007(a) | 0.224 +/- 0.008(b) | 0.221 +/- 0.013(b) | 7.85*   | 3    | 0.204 +/- 0.01(b) | 0.178 +/- 0.009a | 0.173 +/- 0.002(a) | 0.211 +/- 0.019(b) |
| lag-phase  | 9.22** | 3  | 15.38 +/- 0.01(b) | 16.07 +/- 0.01(b) | 13.25 +/- 0.01(a) | 13.79 +/- 0.01(a) | 11.45*  | 3    | 14.52 +/- 0.01(a) | 16.65 +/- 0.01(b) | 16.57 +/- 0.0(b) | 13.59 +/- 0.02(a) |
| JO2        | 181.55*** | 1   | 1.07 +/- 0.13(a) | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| JO2max     | 66.10*** | 1   | 1.67 +/- 0.26(a) | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| JO2ATP     | 155.63*** | 1   | 0.78 +/- 0.15(a) | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| basal JO2  | 35.87*** | 1   | 0.29 +/- 0.06(a) | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| JO2ATP/JO2 | 1.37    | 1    | 0.73 +/- 0.07    | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| c<1        | 133.9*** | 1   | 5.8 +/- 0.82(b) | ND | ND | ND | 42.88* | 1    | 30.2 +/- 0.28(b) | ND | 24.8 +/- 1.13(a) | ND |
| b          | 0.01    | 1    | 14.12 +/- 2.02   | ND | ND | ND | 256* | 1    | 10.2 +/- 0.96    | ND | 7 +/- 0       | ND |
| a+aO3      | 7.63*   | 1    | 6.38 +/- 1.8(a) | ND | ND | ND | 16.79 | 1    | 10.6 +/- 0.85    | ND | 7.4 +/- 0.71   | ND |

Significance of the ANOVA (strain effect) is indicated as follow: * significant at 5%; ** significant at 1%; *** significant at 0.1% (Benjamini-Hochberg correction for multiple testing). df stands for degree of freedom. When ANOVA is significant, Duncan’s class for each strain is noted in bracket. The units are as follow:

- JO2 in nmol of O2 consumption per minute per 10^6 cells
- JO2ATP in pmol/mg dry weight
- JO2ATP/JO2 in % JO2

whatever the mtDNA genotype, harboured similar fermentative features for all ten fermentative parameters, suggesting that mitochondrial genotype has a negligible effect, if any, in fermentation conditions.

**Discussion**

Mitochondrial PCR-based Markers: A Useful Tool for Future Research

Previous mitochondrial genotyping in yeast was based mostly on mtDNA restriction patterns, which is time-consuming and unsuitable for phylogenetic comparison and recombination studies. Mitotyping mtDNA of inter-specific independent hybrids revealed a few events of mtDNA recombination: for one inter-specific hybrid (WU12) no recombinant mtDNA was found, DU23 hybrid was associated with two stable cases of mtDNA recombination. Although the number of tested hybrids is too low to compare accurately the probability of mtDNA recombination between crosses, these results suggest that mtDNA recombination may vary depending on parental strains. In any case, our work provides new molecular tools (PCR-based markers) that will be useful to determine the level of mtDNA recombination. Mitochondrial DNA genotyping could now be applied to other hybrids including other *Saccharomyces* inter-specific hybrids but also intra-specific hybrids of *S. cerevisiae*, in order to assess the mtDNA variation according to the genetic backgrounds. In addition, the use of these PCR-based markers may be useful to definitely resolve whether the fixation of one mitotype is stochastic or not in yeast, as different works suggested either random mitochondrial inheritance [41,42] or non-stochastic one [38,43].

Isogenic Yeast Strains Differing Only for mtDNA: An Original Material to Unravel Nucleo-mitochondrial Interactions and Mitochondrial Impact

Previous work addressed the relationships between mtDNA variation and phenotypic traits through the study of reciprocal hybrids in various organisms such as plants [29–32], insects [33], birds [34] and fishes [35]. However, in most of these cases, the phenomenon of parental genomic imprinting may be confounded with the effect of mtDNA variability [36]. Here, we exploited the peculiar mtDNA inheritance in yeast to produce hybrids being fully isogenic at the nuclear level, but possessing either Sc-mtDNA or Su-mtDNA. Such a biological material is particularly appropriate for the proper testing of the phenotypic impact of mtDNA polymorphism, in absence of reciprocal parental imprinting. In addition, hybrids differing only for mtDNA could be useful for future investigations regarding nucleo-cytoplasmic interactions. Previous works in yeast revealed nucleo-mitochondrial epistasis in yeast, with phenotypic effect on fitness [76]. Incompatibility between *S. cerevisiae* mitochondria and a nuclear gene of *S. bayanus*
Figure 4. Growth dynamics under respiratory conditions for WU12 and DU23 interspecific hybrids. Population growth was assessed on YPEG medium, using flow cytometry. For each strain, triplicates were made and error bars show standard deviations. The growth kinetics are represented in small captions, while large captions focus on the first part of growth dynamics.

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AEP2 was shown to be responsible for hybrid sterility [77]. Additional ‘incompatibility’ genes were further identified within Saccharomyces hybrids of S. cerevisiae, S. bayanus and S. paradoxus [78]. The relationship between cytonuclear incompatibilities and hybrid sterility suggests that this mechanism may be involved in reproductive isolation and subsequently in speciation [79].

Table 4. Results of the ANOVAs: F values and Mean values for fermentation parameters.

| WU12 interspecific cross | Mean value +/- SD (Duncan's class) | DU23 interspecific cross | Mean value +/- SD (Duncan's class) |
|-------------------------|-----------------------------------|--------------------------|-----------------------------------|
| **ANOVA**               | Fvalue | df | WU1-1 Su | WU1-2 Su | WU1-9 Sc | WU1-2-8 Sc | WU1-2-9 Sc | Fvalue | df | DU2-1 Sc | DU2-3 Sc | DU2-4 Sc | DU2-9 Sc |
| Ethanol                 | 0.49   | 3  | 10.97 +/- 0.08 | 11.07 +/- 0.15 | 10.94 +/- 0.2 | 11.03 +/- 0.12 | 1.91   | 3  | 11.00 +/- 0.17 | 10.88 +/- 0.11 | 11.15 +/- 0.17 | 11.1 +/- 0.14 |
| residual sugar          | 0.21   | 3  | 2.00 +/- 2.77 | 1.47 +/- 1.29 | 0.77 +/- 0.64 | 1.67 +/- 2.37 | 2.92   | 3  | 3.93 +/- 4.11 | 6.37 +/- 1.11 | 0.53 +/- 0.42 | 1.37 +/- 1.36 |
| acetic acid             | 0.81   | 3  | 0.05 +/- 0.03 | 0.07 +/- 0.06 | 0.09 +/- 0.01 | 0.09 +/- 0.02 | 7.44   | 3  | 0.08 +/- 0.03 | 0.04 +/- 0.04 | 0.05 +/- 0.04 | 0.17 +/- 0.05 |
| Glycerol                | 0.35   | 3  | 11.2 +/- 0.8  | 10.9 +/- 0.6  | 11.2 +/- 0.6  | 11.4 +/- 0.7  | 0.83   | 3  | 9.7 +/- 0.9  | 9.3 +/- 0.8  | 10.1 +/- 1.0 | 10.3 +/- 1.0  |
| CO2max                  | 0.63   | 3  | 86.78 +/- 2.04 | 86.55 +/- 0.28 | 87.89 +/- 1.17 | 87.25 +/- 1.04 | 4.61   | 3  | 86.35 +/- 2.97 | 84.4 +/- 0.26 | 88.89 +/- 0.75 | 87.95 +/- 0.77 |
| lag phase               | 6.07   | 3  | 38.0 +/- 1.8  | 42.5 +/- 4.3  | 35.9 +/- 5.8  | 40.7 +/- 3.3  | 1.94   | 3  | 26.4 +/- 2.1  | 27.6 +/- 2.2  | 28.2 +/- 0.3  | 24.3 +/- 2.2  |
| AF time                 | 2.12   | 3  | 126.0 +/- 5.0 | 117.5 +/- 3.4 | 110.2 +/- 5.6 | 106.2 +/- 14.6 | 0.84   | 3  | 158.0 +/- 15.9 | 150.3 +/- 0.3 | 165.5 +/- 8.6 | 160.6 +/- 2.4  |
| Vmax                    | 1.39   | 3  | 1.26 +/- 0.02 | 1.41 +/- 0.15 | 1.30 +/- 0.04 | 1.41 +/- 0.09 | 2.08   | 3  | 1.19 +/- 0.08 | 1.14 +/- 0.06 | 1.05 +/- 0.03 | 1.14 +/- 0.05 |

Significance of the ANOVA (strain effect) is indicated as follow: * significant at 5%; ** significant at 1%; *** significant at 0.1% (Benjamini-Hochberg correction for multiple testing). df stands for degree of freedom. When ANOVA is significant, Duncan’s class for each strain is noted in bracket. The units are as follow: ethanol in percent volume, residual sugar in g L⁻¹, acetic acid in g L⁻¹, glycerol in g L⁻¹, CO2max in g L⁻¹ h⁻¹, lagphase and AF time in h, Vmax in g CO₂ L⁻¹ h⁻¹.

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Nucleo-mitochondrial interactions (also designed as mitonuclear interactions) may have also played a major role in other evolutionary processes, like in the evolution of sex [80]. Therefore, hybrids differing only for mtDNA may help understanding the role played by cytonuclear interactions in yeast evolution and adaptive ability.

Mitochondrial DNA Polymorphism has a Phenotypic Impact on Respiration, not on Fermentation

We showed that under fermentative conditions, no phenotypic differences were observed between hybrids having either Sc-mtDNA or Su-mtDNA. It was suggested that mitochondria may play a role in fermentation, in particular because trace amounts of oxygen are necessary for completing fermentation [44], particularly under high sugar concentrations. However, it has been shown that under these conditions, oxygen was not consumed by mitochondria but used for sterol biosynthesis and NADPH-dependent systems localized in microsomal membranes [81]. It should be noted that the fermentative conditions used here were permissive (for oenological conditions), with normal-to-low sugar content (188 g/L). It is possible that under harsher fermentative conditions we may have observed significant differences between hybrids having different mitotypes. Additional analyses under various fermentative environments, from permissive to harsh, will help determining whether mtDNA variation may affect fermentation parameters.

By contrast, under respiratory growth conditions, large differences were associated with the mitotypes. This result is not surprising, knowing that the replacement of the mitochondria of one "Saccharomyces" species by another is usually associated with variation in traits related to respiration [75,82,83]. Here, we showed that hybrids having Sc-mtDNA start to grow earlier and faster than their counterparts with Su-mtDNA. The differences in population growth could be related to the respiratory rate (the higher respiratory rate $\dot{V}O_{2}$, the higher the intrinsic growth rate $r$ and the lower the lag-phase time). Accordingly, previous work showed that the respiratory rate varied greatly from one strain to another and was related to cell growth in S. cerevisiae species [65]. In addition, the differences in respiratory rates between hybrids harbouring either Sc-mtDNA or Su-mtDNA were associated with cytochrome contents variation, particularly with $a+a_3$ content which appears to be higher for Sc-mtDNA than for Su-mtDNA. It has been shown that electron transfer through cytochrome $a+a_3$ is a main controlling step in mitochondrial oxidative phosphorylation in yeast [64,84]. Thus, an increase in cell cytochrome $a+a_3$ content induce a nearly proportional increase in cell respiration during growth. From a bioprocess point of view, the mtDNA inheritance of interspecific hybrids has to be taken into account for selection. In fact, although some industrial starters used in brewing [85] or winemaking [86] are interspecific hybrids, few studies have investigated the role of mtDNA on their aerobic propagation [87]. The respiratory rate discrepancy observed here between Sc-mtDNA and Su-mtDNA is a key factor that likely affects biomass yield of interspecific hybrids and therefore their subsequent development for industry.

Whatever the molecular mechanisms underlying differences in cytochrome contents and thus in respiratory rates, we demonstrated clearly that mitotypes strongly impact cell growth in yeast, and potentially subsequent fitness. To test this last hypothesis, we predicted the evolution of a yeast population initially composed of 1:1 ratio of Sc-mtDNA:Su-mtDNA cells. Using the cell growth parameters calculated through logistic fit, we showed that after four recurrent in silico cultures with initial population size of $10^6$ cells per mL, the Sc-mtDNA mitotype outcompeted Su-mtDNA mitotype and represented 92.9% of the total population for WU12 and 96.5% for DU23 respectively (Figure 6). Far from the hypothesis that mtDNA variation is neutral, our work shows that mitochondrial polymorphism can have strong impact on fitness components and hence on the evolutionary fate of the yeast.

![Figure 5. Fermentation kinetics in Sauvignon grape must for WU12 and DU23 interspecific hybrids.](doi:10.1371/journal.pone.0075121.g005)
populations. From these results, we can hypothesize that the environmental conditions could influence mitochondrial inheritance in interspecific hybrids: under fermentative conditions, hybrids may fix stochastically one or the other mt-DNA, while respiratory environments may increase the probability to fix Sc-mtDNA. The interaction with environments may explain why mitochondrial inheritance was described either as random [41,42] or non-stochastic [38,43] in previous works. In any case, our work provides both the biological material and the genetic markers necessary to elucidate the mechanisms of mitochondrial inheritance.

Supporting Information

Figure S1  Microscopy of WU12 interspecific hybrids harboring either Sc-mtDNA or Su-mtDNA. Several mitochondria per cell are observable (black arrows). The number of mitochondria, their volume, and the number of cristae are similar for both mitotypes. (TIF)

Table S1  Development of polymorphic mitochondrial markers. a SGD [http://www.yeastgenome.org]; b GenBank: EU852811.1 [73]; c range observed for 12 S. cerevisiae strains; d range observed for 4 S. uvarum strains; e RFLP: Restriction Fragment Length polymorphism, LP: Length Polymorphism of amplicon. (XLSX)

Table S2  mtDNA inheritance for two inter-specific crosses between S. cerevisiae x S. uvarum. ND: not detected. (XLSX)

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Author Contributions

Conceived and designed the experiments: WA IMP DdV DS MB PM. Performed the experiments: WA TdS MR BS PM. Analyzed the data: WA MR PM. Wrote the paper: WA MR IMP DdV DS PM.

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Figure 6. Theoretical evolution of mixed-populations with different mitotypes under respiratory conditions. Modeling population growth was made using the kinetics parameters (maximal population size $K$, intrinsic growth rate $r$ and lag-phase) calculated under respiratory conditions (YPEG medium). The initial mixed population contained $10^6$ cells per mL (ratio 1:1 Sc-mtDNA:Su-mtDNA). When the maximal population size was reached (grey dashed line), the next cycle started with $10^6$ cells per mL. After four cycles, the Sc-mtDNA mitotype represented 92.9% of the total population for WU12, and 96.5% for DU23.
doi:10.1371/journal.pone.0075121.g006

Table S1  Development of polymorphic mitochondrial markers. a SGD [http://www.yeastgenome.org]; b GenBank: EU852811.1 [73]; c range observed for 12 S. cerevisiae strains; d range observed for 4 S. uvarum strains; e RFLP: Restriction Fragment Length polymorphism, LP: Length Polymorphism of amplicon. (XLSX)

Table S2  mtDNA inheritance for two inter-specific crosses between S. cerevisiae x S. uvarum. ND: not detected. (XLSX)

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Author Contributions

Conceived and designed the experiments: WA IMP DdV DS MB PM. Performed the experiments: WA TdS MR BS PM. Analyzed the data: WA MR PM. Wrote the paper: WA MR IMP DdV DS PM.

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Figure 6. Theoretical evolution of mixed-populations with different mitotypes under respiratory conditions. Modeling population growth was made using the kinetics parameters (maximal population size $K$, intrinsic growth rate $r$ and lag-phase) calculated under respiratory conditions (YPEG medium). The initial mixed population contained $10^6$ cells per mL (ratio 1:1 Sc-mtDNA:Su-mtDNA). When the maximal population size was reached (grey dashed line), the next cycle started with $10^6$ cells per mL. After four cycles, the Sc-mtDNA mitotype represented 92.9% of the total population for WU12, and 96.5% for DU23.
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Table S1  Development of polymorphic mitochondrial markers. a SGD [http://www.yeastgenome.org]; b GenBank: EU852811.1 [73]; c range observed for 12 S. cerevisiae strains; d range observed for 4 S. uvarum strains; e RFLP: Restriction Fragment Length polymorphism, LP: Length Polymorphism of amplicon. (XLSX)

Table S2  mtDNA inheritance for two inter-specific crosses between S. cerevisiae x S. uvarum. ND: not detected. (XLSX)

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