Restoring fertility in yeast hybrids: Breeding and quantitative genetics of beneficial traits

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Hybrids between species can harbor a combination of beneficial traits from each parent and may exhibit hybrid vigor, more readily adapting to new harsher environments. Interspecies hybrids are also sterile and therefore an evolutionary dead end unless fertility is restored, usually via auto-polyploidisation events. In the *Saccharomyces* genus, hybrids are readily found in nature and in industrial settings, where they have adapted to severe fermentative conditions. Due to their hybrid sterility, the development of new commercial yeast strains has so far been primarily conducted via selection methods rather than via further breeding. In this study, we overcame infertility by creating tetraploid intermediates of *Saccharomyces* interspecies hybrids to allow continuous multigenerational breeding. We incorporated nuclear and mitochondrial genetic diversity within each parental species, allowing for quantitative genetic analysis of traits exhibited by the hybrids and for nuclear–mitochondrial interactions to be assessed. Using pooled F12 generation segregants of different hybrids with extreme phenotype distributions, we identified quantitative trait loci (QTLs) for tolerance to high and low temperatures, high sugar concentration, high ethanol concentration, and acetic acid levels. We identified QTLs that are species specific, that are shared between species, as well as hybrid specific, in which the variants do not exhibit phenotypic differences in the original parental species. Moreover, we could distinguish between mitochondria-type–dependent and –independent traits. This study tackles the complexity of the genetic interactions and traits in hybrid species, bringing hybrids into the realm of full genetic analysis of diploid species, and paves the road for the biotechnological exploitation of yeast biodiversity.

hybrids | QTL | yeast | breeding

Hybridization is important evolutionarily as well as industrially, as it may offer advantageous gene combinations and traits of interest to the newly formed hybrids. Hybrids between species are commonly found in both natural and domestic situations with as many as 25% of plant species and 10% of animal species hybridizing naturally (1). The genus *Saccharomyces* encompasses eight species, included the newly discovered *Saccharomyces jurei* (2, 3), which all readily hybridize among them. In the *Saccharomyces* yeasts, there are many examples of hybrids from both natural (wild) as well as fermentation sources, and indeed, as many as 10% of all yeast isolates are hybrids (4–6). The stringent condition of beer and wine fermentations in particular represent a fertile ground for hybrids, influencing their creation, stabilization, and phenotypic and genetic makeup (7, 8). Indeed, *Saccharomyces pastorianus* (synonym *Saccharomyces carlsbergensis*), a *Saccharomyces cerevisiae*/*Saccharomyces eubayanus* hybrid, has been employed in beermaking since the 15th century (9, 10). *Saccharomyces cerevisiae*/*Saccharomyces kudriavzevii* hybrids have also been isolated from wine, beer, and cider fermentations along with *S. cerevisiae*/*Saccharomyces uvarum* hybrids and the triple hybrid between *S. cerevisiae*, *S. uvarum*, and *S. kudriavzevii* as well as *S. cerevisiae*, *S. uvarum*, and *S. eubayanus* (11, 12). Moreover, examples of hybrids between the closely related species *S. cerevisiae* and *Saccharomyces paradoxus* have been isolated from wild environments (13). Interspecies hybrids of *Saccharomyces* species have therefore been used as model organisms for studying natural evolution, speciation, and fitness adaptation to different environments.

Recently, much work has gone into the generation of de novo yeast hybrids, exploiting their potential for the production of biofuels (14), brewing (15, 16), and winemaking (17). Interspecies hybrids are not only selected for their capability to combine the advantageous traits of the parent strains, as the genomes from both parents undergo chromosomal rearrangements, mutations, widespread transcriptional changes (18, 19), and gene loss and gene duplications, which also impact the nature of protein complexes formed (20). Hence, new and improved phenotypes can arise thanks to heterosis or hybrid vigor (21).

A great deal has been learned on the acquired properties of particular hybrids from comparative genomics and molecular studies. However, to date, no thorough genome-wide analysis of genetic contributions to traits of industrial interest has been possible. The sterility of the interspecies yeast hybrids is in fact hindering the application of both predictive quantitative approaches and any attempt of strain improvement via breeding. A

Significance

Interspecies hybrids, for which mules are a common example, are sterile and therefore an evolutionary dead end. Hybrid sterility has been an obstacle to classical genetic analysis, predictive quantitative approaches, and attempts at strain improvement via breeding. Here, we overcame infertility by creating hybrid tetraploids of yeast species to allow continuous multigenerational breeding. Thus, by exploiting interspecific genetic diversity, we were able to create an unprecedented number of meiotic progenies with different combinations of traits. We showed that the offspring of different hybrids have extreme phenotypes, identified quantitative trait loci (QTLs) dependent of the mitochondria, and discovered QTLs that are uniquely generated in hybrids and for which the allelic variation has no phenotypic consequences in the parental species.

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previous study by Greig et al. (22) showed that sterility of interspecies yeast hybrids can be overcome by creating tetraploids, using a mating type locus (MAT) deletion strategy. The engineered tetraploid hybrids were able to produce viable diploid progeny after undergoing meiosis (22). Another study by Schwartz et al. (23) demonstrated the ability to mate a hybrid for quantitative trait loci (QTL) analysis using expression of *HO* to switch a diploid nonmater to a mater. QTL mapping in particular has shown to be a powerful tool for understanding the genetic basis of various complex traits and has been similarly applied in industrial and medical applications (24, 25). Nonetheless, QTL analysis and understanding of hybrid genetics remains poor and limited to a single generation of meiosis in these studies, due to the sterility of *Saccharomyces* hybrids.

In this study, we used two different approaches to bring de novo yeast hybrids into the realm of genetic analysis and improvement by breeding. A large set of de novo hybrids was engineered by crossing geographically distinct *Saccharomyces* strains of different species. We were able to generate genetically and phenotypically diverse populations through multiple rounds of interbreeding. Diploid F2 and F12 progeny were phenotyped for several traits, and two approaches toward genotyping pools of phenotypic extremes were used to map genetic variation in both parental genomes responsible for the phenotypes selected. For three sets of hybrids, F12 diploid hybrid progeny were arrayed and phenotyped under several different conditions. For each hybrid, two versions were assessed, one each with each parental species mitochondrion. The top 20 and bottom 20 from an array of 384 were pooled for sequencing and the frequencies of segregating single nucleotide polymorphism (SNP) sites in the two parental species genomes were compared to identify QTLs involved in the traits. For one of these hybrids (two mitochondrial versions), strong selection was applied to a population of 10^8 F12 progeny resulting in fewer than 10^6 survivors whose pooled genomic DNA was compared to the pool prior to selection, for several conditions. We demonstrate *Saccharomyces* hybrids are now amenable to all the tools available for yeast, including breeding and utilization of the vast genetic diversity available. We show that there are QTLs both unique to one parent species or shared by both, QTLs-dependent and -independent of the mitochondrial origin, and QTLs that are specific to the hybrid and not to the parent species where the variants are segregating.

Results

Construction of Tetraploid Yeast Hybrids from a Variety of Parental Strains Leads to Restoration of Fertility. We constructed yeast hybrid tetraploid lines combining four different genomes of strains coming from two separate species belonging to the *Saccharomyces* genus. To construct such fertile tetraploid hybrids, we employed two different strategies (Fig. 1). First interspecies diploid hybrids were constructed using stable haploids after deletion of *HO* (five for *S. cerevisiae*/*S. jurei* and 10 for *S. cerevisiae*/*S. kudriavzevii*, *SI Appendix*, Table S1), followed by reciprocal deletion of the MAT locus, and subsequent crossing of the two diploid mater hybrids. In the second strategy, intraspecies diploid strains were first constructed by crossing diverged populations of the same species, again using stable haploids after deletion of *HO* (10 *S. cerevisiae*, six *S. uvarum*, and two *S. eubayanus* intraspecies crossings, *SI Appendix*, Table S2), followed by reciprocal deletion of the MAT locus and subsequent hybridization of the two diploid maters. Tetraploid lines of *S. cerevisiae*/*S. kudriavzevii* (Sc/Sk) and *S. cerevisiae*/*S. jurei* (Sc/Sj) hybrids were created with this first strategy while *S. cerevisiae*/*S. uvarum*, and *S. cerevisiae*/*S. eubayanus* were constructed using the second approach.

Hybrids between *Saccharomyces* species are homoplasmic and tend to carry mitochondrial DNA from only one parent. The natural hybrid *S. pastorianus* (hybridized from *S. cerevisiae* and *S. eubayanus*) carries the *S. eubayanus* mitochondria (26, 27), while other industrial hybrids of *Saccharomyces* species used in wine and cider production have retained *S. cerevisiae* mitochondrial genome (28). It has also recently been shown that the type of mitochondria inherited affects the phenotype (29–31) and the transcriptional network (18) in hybrids. Therefore, here, each of the tetraploid hybrids constructed were also selected for different mitotypes. Throughout the paper, in the hybrid nomenclature, a subscripted $_m$ following the initial of the species represents the particular mitochondria inheritance of that hybrid (i.e., Sc_m/Sj is the tetraploid hybrid containing Sc mitochondria, and Sc/Sj_m is the tetraploid hybrid containing Sj mitochondria).

A total of 226 tetraploid hybrids were created, and each hybrid had four unique parental strains and a unique mitochondrion contributing to the genome (Dataset S1). All the constructed tetraploids were fertile as they had a homologous set of chromosomes to align in meiosis. While diploid hybrids between species of *Saccharomyces* genus are reproductively isolated (3, 4), the tetraploid hybrids constructed here were fertile and exhibited spore viability as high as 98% (*SI Appendix*, Table S3) as has previously been reported for tetraploids (22). Ultimately, the diploid F1 spores obtained from the tetraploid hybrids were sequentially randomly mated and sporulated 11 times until the F12 generation (Fig. 1). From the F12 generation around 384 spores for each were isolated for further studies.

Meiotic Offspring of Tetraploid Hybrids Exhibit Broad Phenotypic Diversity. Intercrossing different populations of *Saccharomyces* species over many generations reduces linkage disequilibrium by increasing recombination. To assess whether the fitness traits are associated with genetic linkage, we assessed the phenotypic landscape of F1 and F12 diploid generations in up to 12 conditions encompassing different growth temperatures, carbon sources, and stressors. An example of phenotypic divergence between F1 diploid segregants of Sc/Sj (Sc^{OS3}/Sj^{D5088}/Sc^{OS104}/Sj^{D5095}) and Sc/Sk (Sc^{OS253}/Sk^{OS575}/Sc^{OS104}/$Sk^{IFO1802}$) tetraploids harboring different mitotypes is reported in *SI Appendix*, Fig. S1. Significant fitness differences were seen in all the segregant lines with a dispersion up to 0.33 (quartile coefficient of dispersion, *SI Appendix*, Table S4), with some progeny being fitter than any of the parents (*SI Appendix*, Fig. S2) and some being less fit (transgressive variation) in virtually all cases.

When colony size is normalized within each specific condition, to allow the teasing apart of fitness differences between spores derived from the same tetraploid line, F12 segregants of Sc_m/Sj, Sc/Sj_m, Sc_m/Sk, and Sc/Sk_m again exhibited a large phenotypic variation in all the tested conditions (Fig. 2).

Given that the tetraploid lines are composed of *S. cerevisiae* combined with genomes of other *Saccharomyces* species with different levels of phylogenetic distance, we investigated whether such differences in genome divergence have an impact on phenotypic plasticity in any given condition. By analyzing the unnormalized fitness data, to tease apart differences in the colony size range between progeny from separate tetraploid lines, no striking differences were found in either range or dispersion between different hybrids (*SI Appendix*, Fig. S3). Therefore, the different levels of divergence of the genomes present did not impact significantly on phenotypic range and plasticity in the progeny in the hybrid lines generated.

Phenotypic Diversity of Tetraploid Hybrids is Underpinned by the Presence of QTLs. To identify the genetic basis underlying the observed phenotypic diversity, we performed QTL analysis on selected segregant pools of Sc/Sj, Sc/Sk, Sc/Se, and Sc/Su hybrids. Two different methods for QTL analysis were employed. The Multipool technique, pioneered by ref. 32, was used to analyze the F12 generation of Sc/Sj, Sc/Sk, and in Sc/Se hybrids, while the Pooled Selection method, or bulk segregant analysis (33, 34), was applied to Sc/Se and Sc_m/Su hybrids. Both approaches proved

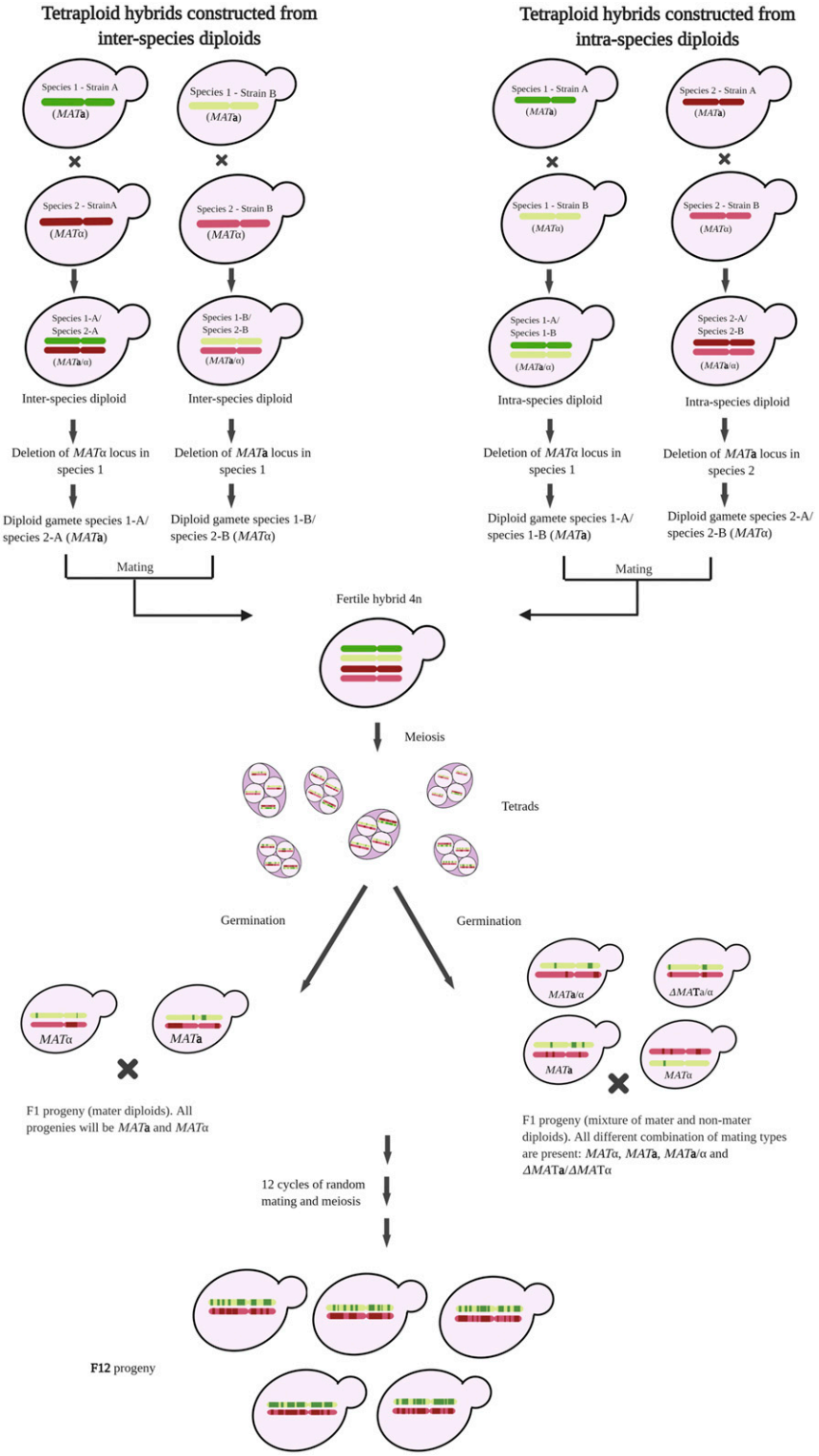

Fig. 1. Construction of fertile hybrids. The tetraploid hybrids were constructed using two different strategies. In the first strategy, two different *Saccharomyces* species were crossed to obtain 2n interspecies diploid hybrids (species 1 and species 2 are represented in shade of green and red, respectively). Two different such hybrids were made to behave as gametes by deleting either the *MAT*a or α locus in one species and subsequently crossed to make the fertile tetraploid hybrid (4n). In the second strategy, two diverged populations (A and B) of the same species were crossed to construct the 2n intraspecies strains. Two different intraspecies lines were also made to behave as gametes by deleting the *MAT*a from species 1 and *MAT*α from species 2, and subsequently, these were crossed to construct the 4n hybrids. The tetraploid hybrids were sporulated and germinated to obtain the diploid F1 progeny which were randomly mated and sporulated several times until a F12 generation with a high level of scrambled genomes.

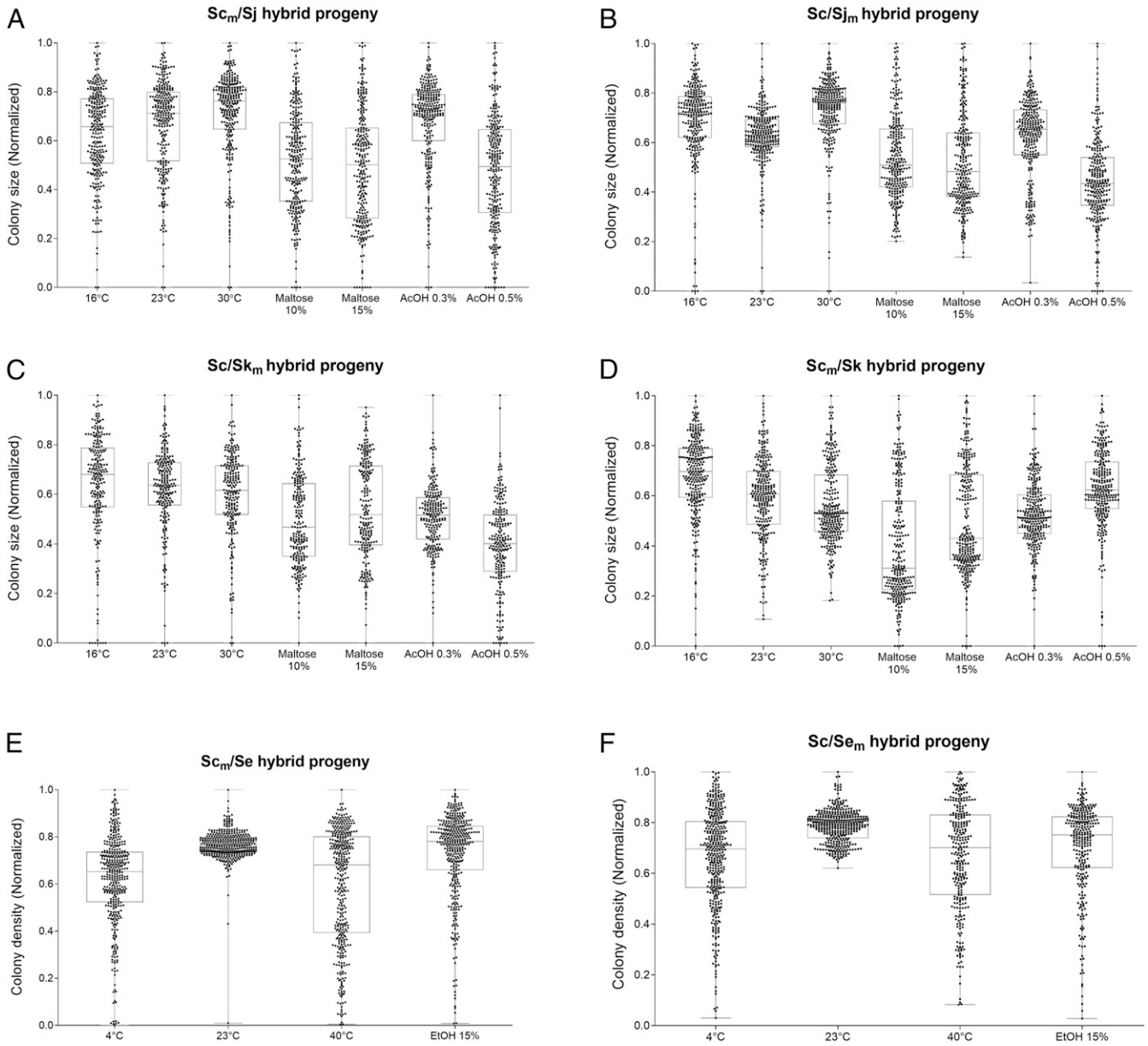

Fig. 2. A box plot of the fitness of F12 diploid progeny for *S. cerevisiae*/*S. jurei* (Sc_m/Sj and Sc/Sj_m) and *S. cerevisiae*/*S. kudriavzevii* (Sc_m/Sk and Sc/Sk_m) and *S. cerevisiae*/*S. eubayanus* (Sc_m/Se and Sc/Se_m) hybrids. For Sc_m/Sj (*A*), Sc/Sj_m (*B*), Sc_m/Sk (*C*), and Sc/Sk_m (*D*), the normalized colony size was used as proxy of fitness (see *Materials and Methods*) and was scored in YPD at different temperatures 16 °C, 23 °C, and 30 °C in YP- Maltose (10% and 15%) and in YPD with 0.3% and 0.5% acetic acid (AcOH). For Sc_m/Se (*E*) and Sc/Se_m (*F*), the colony optical density was used as proxy of fitness (see *Materials and Methods*) and was scored in YPD at different temperature 4 °C, 23 °C, and 40 °C and in YPD with 15% Ethanol (EtOH). Each black dot represents a different F12 diploid hybrid. The upper and lower error bars represent the minimum and maximum values.

successful in mapping QTL regions in a variety of conditions for all hybrids; however, a higher number of QTLs and more consistent results across the conditions tested were obtained using the Multipool approach (*SI Appendix*, Table S5). A comprehensive list of the QTL intervals mapped, including coordinates of the regions, logarithm of the odds (LOD) scores, and gene content is presented in Dataset S2.

For the Multipool, the top 20 and bottom 20 individual of 384 arrayed were pooled for each condition for comparison. Sc/Sj and Sc/Sk hybrids were selected at low temperature (16 °C), in 15% maltose, and in 0.3% acetic acid, while Se/Sc hybrids were selected in 10% ethanol and low and high temperatures (4 and 40 °C). Such conditions are relevant to fermentation industries. Low temperature is required for the storage of brewing yeast and for fermentation of lager beer. Maltose is one of the key wort sugars, and the high concentration of this sugar mimics the osmotic pressure exerted upon yeast in high gravity wort (35). Acetic acid is found in grape must, and wine-producing yeasts are known to require the resistance to this stress (35, 36). Ethanol is the major stressor for both the production of fermented beverages and bioethanol (35).

From segregants generated from the tetraploids Sc_m/Sj and Sc/Sj_m, a total of 56 QTLs were identified in the *S. cerevisiae* genomes with an average length of 19.4 kb (*SI Appendix*, Table S6). Despite the high similarity between the two *S. jurei* parental strains (2), we were able to map 62 QTL regions in the genome of this species. However, with an average length of 35.5 kb, the QTL mapping intervals were the longest observed in the hybrids

generated due to the lower density of segregating markers in the two genomes of this species.

An even higher number of QTLs was detected in the progeny of Sc_m/Sk and Sc/Sk_m tetraploids with up to 155 and 128 regions mapped in *S. cerevisiae* and *S. kudriavzevii*, respectively (*SI Appendix*, Table S6). Here, the QTL intervals were narrower than those seen in the *S. jurei* genome, with only 15.6 kb average length on *S. cerevisiae* alleles and 18.6 kb on *S. kudriavzevii* ones, as there is a higher density of segregating markers in these genomes.

Similar results were obtained in Sc_m/Se and Sc/Se_m hybrids analyzed for their fitness in high ethanol concentration and at high and low temperatures (40 and 4 °C) (*SI Appendix*, Table S6). Here, we were able to identify 111 and 64 QTLs regions in *S. cerevisiae* and *S. eubayanus*, respectively, with an average length of 18.5 kb and 21.82 kb.

A total of 28 genes mapped in different QTLs in Sc/Sj and Sc/Sk hybrids were classified as potential causal genes, as their role in the selection condition was already confirmed by previous published work (37) (*SI Appendix*, Table S7). For example, one of the acetic acid QTL detected in *S. cerevisiae* chromosome III (52 to 97 kb) in Sc_mSj hybrids contains variant alleles of *LEU2*. The gene encodes for a β-isopropyl-malate dehydrogenase, and null mutants are reported as sensitive to acetic acid while its overexpression increase acetic acid resistance (38).

Among the genes identified, a total of 43 genes with segregating alleles found in low-temperature QTLs were previously identified in large-scale competition studies carried out in *S. cerevisiae* at 16 °C, with an additional five being described as cold favoring by thermodynamic model predictions (*SI Appendix*, Table S7) (39).

Thanks to the abundance of data on heat and ethanol sensitivity in *S. cerevisiae* (34, 40–42), a high number of potential causal genes with segregating variation were identified in the QTL regions of Sc/Se hybrids. Thus, we were able to identify up to 38 genes in the 44 QTL regions for the *Sc* alleles that likely promote a fitness advantage while growing at 40 °C (*SI Appendix*, Table S7). Among these, *IRA1*, a regulator of the RAS pathway, was previously validated as a heat-QTL in OS3/OS104 crosses (34). Moreover, two additional genes involved in the RAS/cAMP signaling pathway (*ESB1* and *GPB2*) were mapped in heat QTLs, supporting its involvement in in mediating heat resistance as previously suggested by Parts et al. (34).

The potential causal genes detected in *S. cerevisiae* genomes in Sc/Sj, Sc/Sk, and Sc/Se hybrids may contain amino acid variants that are affecting protein function. Hence, we analyzed these genes through SIFT analysis (Sorting Intolerant From Tolerant) to identify nonsynonimous SNPs underlying the observed phenotypic difference between alleles (43, 44). SIFT analysis was carried out on the 82 predicted *S. cerevisiae* causal genes. A strong effect on the protein function was detected in 23% of potential causal genes due to amino acid differences between the *S. cerevisiae* parental strains (*SI Appendix*, Table S8), while ca. 38% of mutations were inferred as tolerated, and for the remaining 39%, no nonsynonymous SNPs at the protein-coding region were detected.

Gene ontology (GO) analysis did not help to narrow down choices of potential causal gene candidates, since the enrichment GO terms were, at a broad level, only generally associated with intracellular membrane-bound organelle, cytoplasm, catalytic activity, and cellular processes in all the conditions.

In total 14, 22, and 11 pleiotropic QTLs were mapped in Sc/Sj, Sc/Sk and Sc/Se hybrids, respectively (Dataset S3). A 7-kb region on the *S. cerevisiae* chromosome XIII was common across all conditions tested for ScSj_m hybrids, but, interestingly, it was not detected for Sc_mSj in any condition tested. This region contains the genes *CLU1* (a subunit of eIF3), *ANY1* (a protein involved in phospholipid flippase), and *HXT2* (a high-affinity glucose transporter). It is possible that the phenotypic effect of variation in these genes depends solely on mitochondrial–nuclear interactions, independent of the condition. *CLU1* is known to play a role in mitochondrial distribution and morphology but it maintains its respiratory function and inheritance (45, 46). The *Δclu1* mutants possess a more condensed mitochondrial mass found at one side of the cell (45). They are haplo insufficient in nutrient-limited media (45, 47) and haplo proficient in phosphorus-limited media (48).

In parallel to the comparison of small pools performed with Multipool, Pooled selection experiments were performed on Sc_m/Se and Sc/Se_m, Sc_m/Su segregants. For the selected pools, the strength of selection was chosen such that ∼0.1% of the population survived the selective condition which was then pooled for comparison to the unselected population. Specifically, Sc_m/Se and Sc/Se_m were selected for a variety of selectable traits of industrial relevance, spanning from high maltose or glucose concentrations (35%) to H_2O_2 treatment (4 mM), and very low temperature (4 °C), while the Sc_mSu hybrid progeny were selected in yeast extract peptone dextrose (YPD) medium at high temperature (40 °C) and YPD with levulinic acid (50 mM), acetic acid (0.35%) at 23 °C.

The linkage analysis on the Pooled selection segregants yielded narrow intervals, averaging at 18.1 kb and 20 kb in Sc/Se and Sc_m/Su hybrids, respectively. However, only a limited number of QTLs were identified, except for YP-Maltose (*SI Appendix*, Table S5). No QTLs were found among segregating *S. uvarum* alleles (*SI Appendix*, Table S9).

In total, 35 of the 41 QTL regions detected in Sc_m/Se and Sc/Se_m segregants were present in hybrids with a fitness advantage in high maltose concentrations (*SI Appendix*, Table S9). However, it was unexpected that only one QTL was identified when selection took place at 4 °C, given that in the Multipool analysis in the same condition, 45 QTLs were identified. Characteristics such as growth at low temperature, which, albeit selectable, do not show extremes phenotypes, are better discriminated using the Multipool approach as it exploits the richness of fitness data acquired through individual phenotyping. Growth at 4 °C may not be strong-enough selection (i.e., doesn't kill the majority of individuals in the population), resulting in less discrimination in the Pooled selection approach.

Similarly, Pooled selection of Sc_m/Su segregants yielded only nine intervals of interest, all mapping to *S. cerevisiae* alleles across the four conditions tested. These regions included a single interval conferring tolerance to acetic acid, four conferring selective advantage at high temperature, and four giving tolerance in an environment containing levulinic acid. As mentioned above, none were found in the *S. uvarum* genome.

As with Multipool QTL, we identified several genes, which had already been reported as having a phenotypic effect or a function closely linked to the condition tested. For Sc_m/Se and Sc/Se_m hybrids, we detected eight genes in the Pooled Segregants in high maltose concentrations, in which deletion was reported to cause osmotic stress sensitivity (*SI Appendix*, Table S10). Among these, *SKO1*, a basic leucine zipper transcription factor mapped in *S. eubayanus* alleles of Sc/Se_m segregants, has been described having a major role in mediating HOG pathway–dependent osmotic regulation (49).

Overall, our results indicate that the Multipool approach with individuals at each extreme of a phenotype distribution is more efficient than a highly selected pool approach. However, the highly selected pool approach can identify rare genotypes of linked recombinant variants that are too rare to be in the arrays used to choose individuals for the Multipool approach.

Different Types of Mitochondria Have a Profound Effect on the QTL Landscape. Mitochondrial–nuclear interactions have been reported as having a major role in phenotypic variation both in intraspecies and interspecies yeast hybrids (18, 30, 31, 50), affecting respiration (29), fermentation properties (18, 51), progeny fitness (52, 53), reproductive isolation (50), and nuclear transcription (18). Given the complexity and the diversity of the hybrid background, thorough mapping of the epistasis in genome-wide studies

has been a challenge. Here, in order to evaluate how the mitochondria inherited may affect the QTL landscape, we compared QTL regions mapped in diploid hybrid progeny derived from tetraploid lines harboring different mitochondria but the same parental nuclear inputs.

Interestingly, the majority of QTLs detected via the Multipool method were exclusive to a specific mitotype (Fig. 3). In fact, in all of the conditions analyzed, only ca. 2.45% QTL regions (the mean percentage for all hybrids in all the conditions) were in common among the segregants from tetraploid hybrids with different mitochondria. This difference in the QTL landscape between the same yeast hybrid cross, differing only for the mitotype, is consistent with the idea that mitochondrial–nuclear interactions have a genome-wide effect and are important in the context of evolution, as already demonstrated by several studies both in yeast (50, 54) and in other organisms (55–57).

The experiments carried out via Pooled Selection also demonstrated mitotype-specific QTLs. Only one QTL region was in common in the Sc/Se hybrids with different mitochondria: a 21-kb region on chromosome I, containing the genes *FLO1* and *PHO11*, where specific Sc alleles are associated with an increase in fitness at high maltose concentrations. Given these are located in a subtelomeric region, known to be highly variable in copy number and location in addition to sequence as well as difficult to assemble (58, 59), the causal genetic variation may be an unknown sequence linked to the *FLO1* and *PHO11* genes, as these regions are not assembled in all of the genomes utilized here.

Among the QTLs shared between Sc_mSj and $ScSj_m$ segregants, a major maltose QTL near the telomeric regions of chromosome

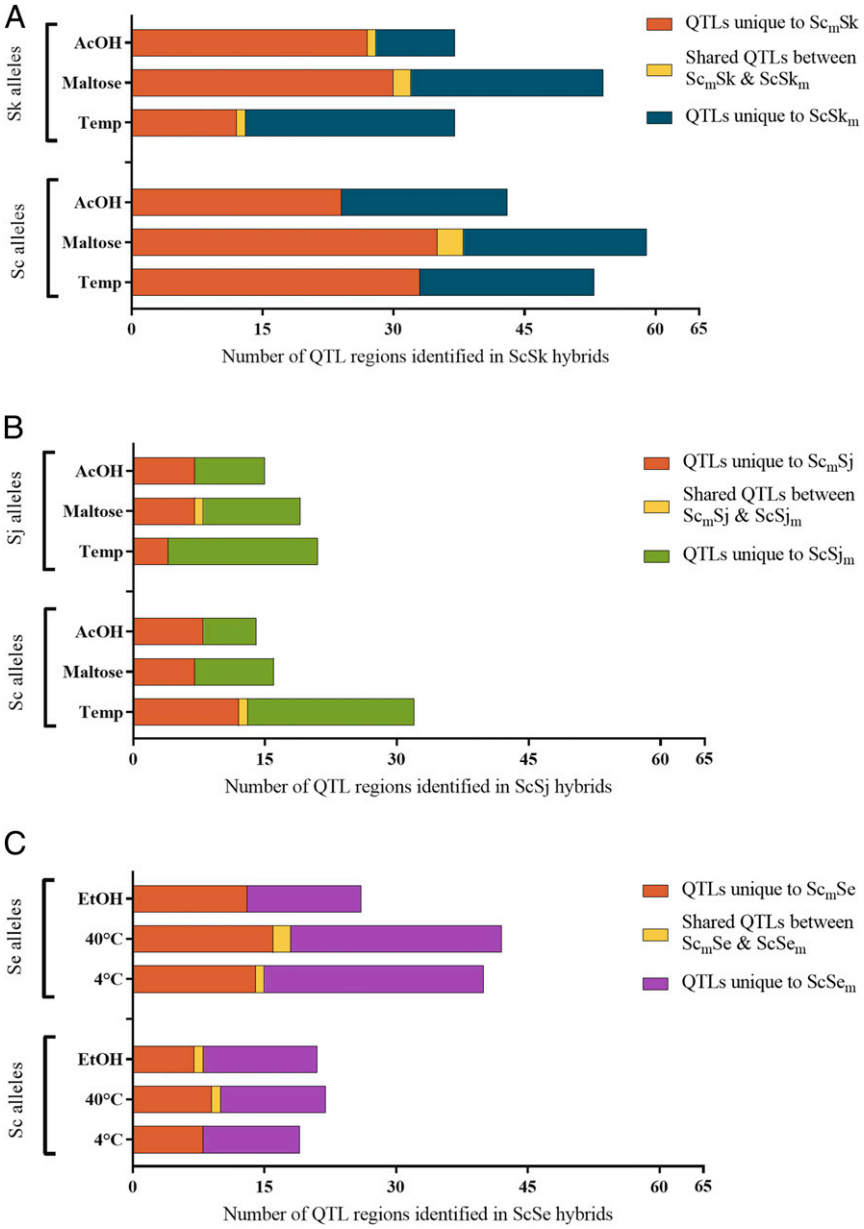

Fig. 3. Hybrids with different mitotype exhibit a different QTL landscape. Boxplot of the distribution of QTLs in *S. cerevisiae*/*S. kudriavzevii* (*A*), *S. cerevisiae*/*S. jurei* (*B*), and *S. cerevisiae*/*S. eubayanus* hybrids (*C*). The QTL regions are grouped first by the alleles and then by growth condition in which they were identified: acetic acid (AcOH), high maltose concentrations (Maltose), low temperature (16 °C or 4 °C), heat, and ethanol (EtOH). The small proportion of QTLs shared between mitotypes of the same hybrid is represented in yellow.

II of *S. cerevisiae* (780 to 795 kb) was detected with a high LOD score (>17). The recurrence of this QTL and its high LOD score could be ascribed to the *MAL3* multigene complex in the subtelomeric region and the natural variation in copy number, location, and sequence of this complex (58). Furthermore, this maltose QTL is also mapped on the *S. cerevisiae* alleles of both Sc_m/Sk and Sc/Sk_m hybrids, pointing to a more general effect of these allelic variants rather than a strain background dependent one.

Overlap of QTL Regions between Different Hybrids Facilitates the Identification of Causal Genes. One way to help to identify relevant genes within QTL regions, which are not specific to a particular hybrid combination, is to investigate overlapping QTL regions detected in different hybrids. Such an approach can lead to the unambiguous identification of genes underlying the phenotypic effect observed.

A total of 12 overlapping intervals, shared by at least three hybrid genomes, were mapped in low-temperature, high-maltose, and high-ethanol QTLs, while an additional 52 intervals were overlapping between two hybrids (Dataset S4). Within acetic acid QTLs, only 12 overlapping intervals were detected with no region shared between more than two species, suggesting a greater diversity of variants connected with the phenotype. Among these intervals, we identified several candidates with biological functions closely linked to the selection condition. For instance, a low-temperature QTL mapped both in *S. cerevisia*e and *S. kudriavzevii* includes *OSH6* (Fig. 4*A*), related to sterol metabolism and, as such, membrane fluidity, often considered a feature of low-temperature adaptation (60). Similarly, an acetic acid QTL mapped in both Sc/Sj_m and Sc_mSk hybrids includes *NQM1*, a nuclear transaldolase involved in oxidative stress response, known to be induced by acetic acid stresses (61) (Fig. 4*B*).

Ethanol and high-temperature QTLs were analyzed only in Sc/Se hybrids, limiting the outcomes compared to the other traits. Nonetheless, we found three overlapping ethanol QTLs with a major region mapped on chromosome XV shared between genomes (Fig. 4*C*). Moreover, this region included the genes *PAC1*, *VPH1*, and *MOD5* in which null mutations are linked to a decreased fitness in ethanol supplemented media (62, 63).

A major overlap was detected in the subtelomeric region of chromosome II, with a maltose QTL shared between the *S. cerevisiae* allele of all Sc/Sj and Sc/Sk hybrids and the *S. jurei* alleles of Sc/Sj_m. The QTL contains a causal gene, *MAL33*, a MAL-activator protein, and two transporters, *CTP1* and *PHO89*, involved in the transport of citrate and phosphate, respectively, which are key metabolites for the glycolytic pathway (Fig. 4*E*).

Remarkably, two low-temperature QTL regions were mapped in at least one mitotype of all tetraploid hybrids analyzed through Multipool. The overlapping regions resulted in both cases in a single-gene intersection with *COQ6* (Fig. 4*F*) and *PEP3* (Fig. 4*D*) identified in four and five different intervals, respectively. *COQ6* is a mitochondrial monooxygenase, which, in addition to its known role in mitochondrial respiration, is involved in fatty acid β-oxidation (64). *PEP3*, instead, is a component of the CORVET membrane-tethering complex, and its role at cold temperature was previously suggested by large-scale competition studies (39). Moreover, SIFT analysis performed with mutfunc (65) predicted a strong deleterious effect of the SNP in the OS253 *PEP3* variant, due to a substitution in a conserved region.

Validation of QTLs via Reciprocal Hemizygosis Analysis. The narrow mapping intervals identified in the Sc/Sk_m hybrid allowed single-gene studies to validate the effect of candidate alleles, which were not strongly identified as causal genes. The fitness of the allelic variants was tested with reciprocal-hemizygosity analysis (RHA) (25) performed on *ASI2*, *FUS3*, and *GIT1*, which are candidate QTLs in acetic acid, low temperature (16 °C), and high maltose, respectively (Fig. 5, *A* and *SI Appendix*, Table S11). Among the genes included in the acetic acid QTL, *ASI2*, part of the Asi ubiquitinase complex, was defined as a potential candidate gene, as its null mutant was previously described as sensitive to oxidative stress in systematic studies (66). *FUS3*, a MAPK protein, was previously described as haplo-insufficient in large-scale competition studies at 16 °C (39). Lastly, we selected the plasma membrane permease *GIT1* included in a high LOD QTL region in chromosome III identified at high maltose concentrations, along *HMRA1*, *HMRA2*, *CDC39*, *CDC50*, and *OCA4*. *GIT1* was deemed the most-promising candidate, as it is involved in phosphate and glycerol-3-phospate transport, important metabolites of the glycolytic pathway (67).

The phenotypic effect of *ASI2*, *FUS3*, and *GIT1* alleles were validated via RHA performed on the hemizygote tetraploid parents (Sc^{OS253}/Sk^{OS575}/Sc^{OS104}/$Sk^{IFO1802}$) in YPD + 0.3% acetic acid at 30 °C, YPD at 10 °C, and YP-Maltose (15%) at 25 °C, respectively (Fig. 5, *B–D* and *SI Appendix*, Table S12). We observed a significant difference of the growth curve integral area (*SI Appendix*, Table S12) between the performance of the allelic variants in all the three genes tested, confirming their impact in the selection condition and validating them as having causal variant alleles. The $FUS3^{OS253}$ and $GIT1^{OS104}$ alleles performed better than the $FUS3^{OS104}$ and $GIT1^{OS253}$ alleles in terms of specific growth rate (*P* value = 0.0010 and 0.0335, respectively) and integral area (*P* value = 0.0047 and 0.0435, respectively) (*SI Appendix*, Table S12), mirroring what we have seen in our Multipool QTL screening. For the *ASI2* gene, the $ASI2^{OS253}$ was the allele prevalent in the high-fitness pool exposed to a high concentration of acetic acid. In the phenotypic validation, the $ASI2^{OS253}$ variant performed worse than the $ASI2^{OS104}$ in terms of integral area (*P* value = 0.0479) and T_{mid} (*P* value = 0.0182) but it reached a higher maximum biomass (*P* value = 0.0001). Although the growth parameters for these two alleles are clearly different, it is more ambiguous whether their fitness performance overlaps with that one detected in the QTL study in which the $ASI2^{OS253}$ was the allele prevalent in the high-fitness pool. This discrepancy could be due to other genes in the close proximity, masking its effect, or to the difference in the phenotypic screening employed, as the F12 segregants were assayed for their growth in solid media. Many of yeast QTLs have shown to be environment and background dependent and have linked sets of quantitative trait nucleotides (QTN) (68–70). In fact, it has been shown that sporulation efficiency in yeast is controlled by four QTNs (71).

Interspecies Hybrids Generate New QTLs Not Present in Parental Intraspecies Crosses. Finally, we investigated whether the phenotypic effect of the *ASI2*, *FUS3*, and *GIT1*, allelic variants, was exclusive to the interspecies hybrid background. If the QTLs are hybrid dependent, then they should not be present in crosses involving only strains belonging to the same species. Hence, we evaluated the presence of QTLs involving different *S. cerevisiae* alleles in intraspecific crosses between the two relevant *S. cerevisiae* strains (Fig. 5, *E–H*). Specifically, the validation was performed through RHA on *S. cerevisiae* Sc^{OS104}/Sc^{OS253} diploids. No significant difference in fitness between the allelic variants was observed for both *ASI2* and *GIT1* alleles, indicating that such QTLs are exclusive to interspecies hybrid background (*SI Appendix*, Table S12). In the Sc^{OS104}/Sc^{OS253} diploids, *FUS3* alleles showed a significant difference in the integral area of the curve (*P* value = 0.0196; *SI Appendix*, Table S12) and growth rate (*P* value = 0.0014, *SI Appendix*, Table S12). This phenotypic variation is, however, opposite to that one observed in Sc/Sk_m tetraploids, suggesting that also in this case the allelic differences are influenced by the interspecies hybrid genomes. Moreover, a temperature QTL including *FUS3* was also mapped in Sc/Sj_m hybrids, suggesting that the phenotypic effect may be indeed hybrid background independent.

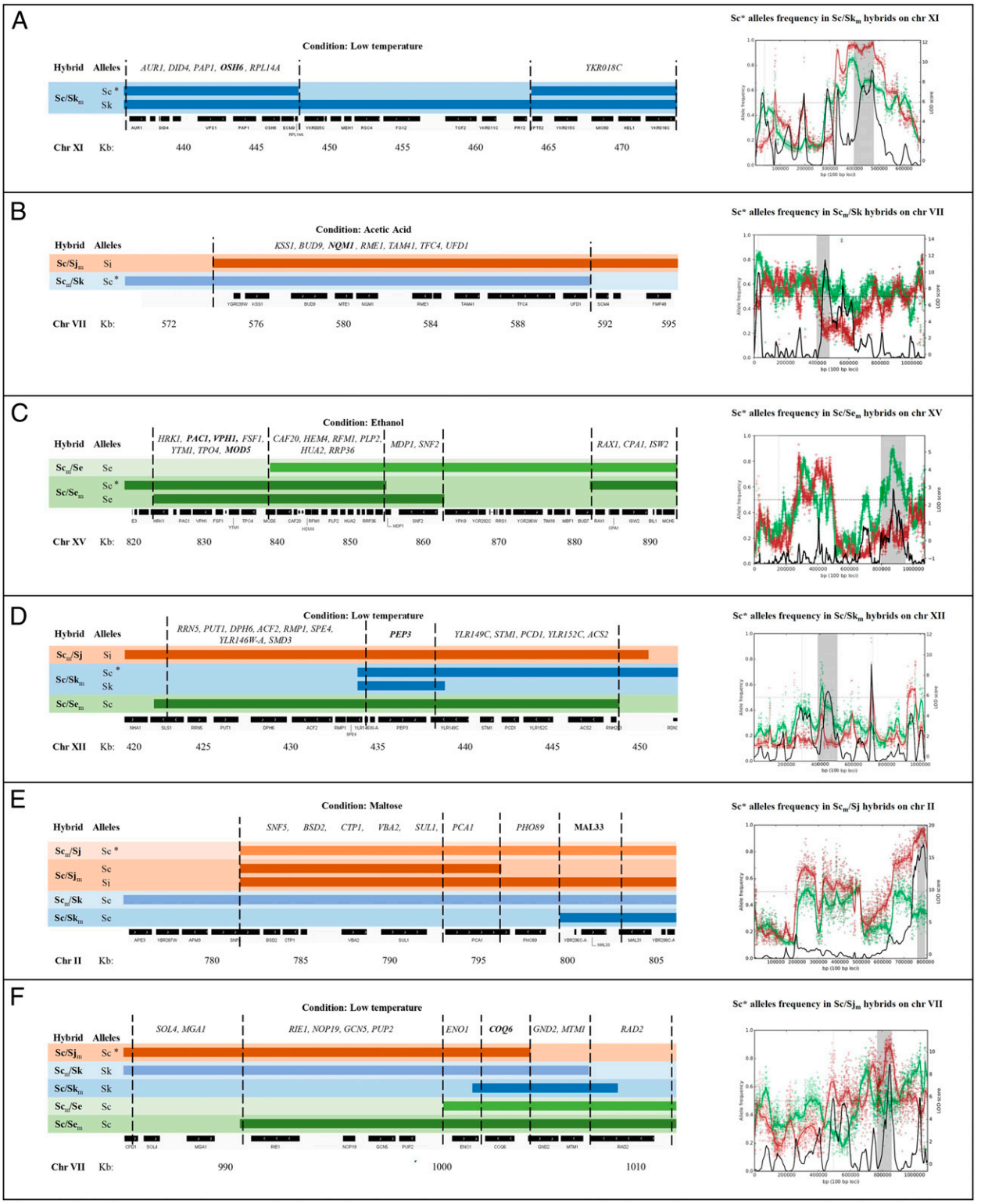

Fig. 4. Example of interspecies QTLs detected in *S. cerevisiae*/*S. jurei*, *S. cerevisiae*/*S. kudriavzevii*, and *S. cerevisiae*/*S. eubayanus* hybrids for low temperature (*A*, *D*, *F*), acetic acid (*B*), ethanol (*C*) and maltose (*E*) traits. The QTL regions, represented by colored bars, are mapped onto *S. cerevisiae* chromosomes to identify the overlapping QTL intervals, and the genes shared between different species and hybrids. The parental species alleles for each hybrid are stated, and the genes in bold denotes potential causal genes. The QTL plots represents the frequency of *S. cerevisiae* alleles, marked in the figure with an asterisk, from different hybrid pool lines. The red and green lines represent the alternative allele frequency of high- and low-fitness pools using the Sc^{OS104} genome as reference. The black line indicates the LOD score, and the gray area is the 90% credible interval of the significance. The dash line is the threshold of LOD considered in this study.

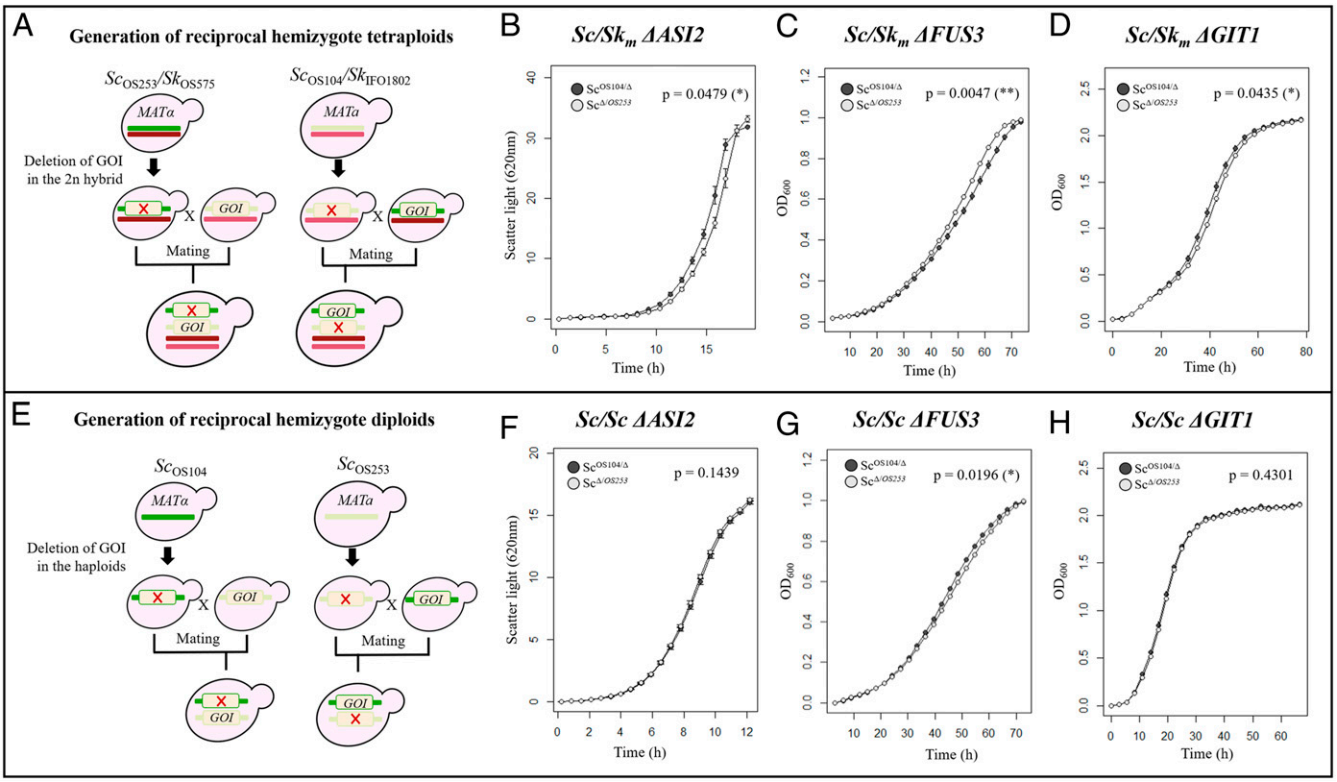

Fig. 5. Validation of the phenotypic effect of candidate genes in inter- and intraspecies hybrid background. Diagram of the construction of the reciprocal hemizygote ScSk$_m$ tetraploid strains (*A*). The gene of interest (GOI) was first deleted from the respective ScSk diploid hybrids. The engineered 2n hybrids were crossed to construct the reciprocal hemizygote tetraploid strains. Growth curves of ScSk$_m$ reciprocal hemizygotes for the *ASI2*, *FUS3*, and *GIT1* genes are shown in *B–D*, respectively. Diagram of the construction of the reciprocal hemizygote diploid strains (*E*). The GOI was deleted from Sc$_{OS104}$ and Sc$_{OS253}$. The engineered haploid strains were crossed to construct the hemizygote diploids. Growth curves of Sc OS104/OS253 reciprocal hemizygotes for the *ASI2*, *FUS3*, and *GIT1* are shown in *F–H*, respectively. Fitness assays were performed in YPD supplemented with 0.3% acetic acid (*B* and *F*), YPD at 10 °C (*C* and *G*), and YP + maltose 15% (*D* and *H*) as outlined in *Materials and Methods*. Significance difference between the integral area of the reciprocal hemizygotes is shown as *P* value assessed by Student's *t* test.

Discussion

Hybrid sterility can be overcome by doubling the genome of the hybrid. Such fertility-restored hybrids, known as amphidiploids, are commonly found in plants and represent the majority of major evolutionary events in angiosperms (72, 73). Tetraploidization, resulting in amphidiploids, can also restore fertility in yeast hybrids (22). *Saccharomyces* yeast hybrids have now entered the realm of classical genetic analysis as well as molecular genetic analysis. Previous studies have created variants of many interspecies hybrids, and some have even been able to complete one round of meiotic recombination, allowing some linkage analysis of genetic variants associated with traits of interest. Here, we take this one step further by overcoming hybrid sterility in ways that allow continuous sequential crossing resulting in advanced intercross lines that bring in the full power of breeding genetics and quantitative genetic analysis of hybrid traits. We demonstrate that multiple traits of interest can be analyzed with the same sensitivity and resolution as performed for intraspecies studies. Moreover, we compare different QTL analysis approaches and can advise that the Multipool approach is more efficient for detecting most QTLs than pools of highly selected subpopulations. The Multipool approach will not resolve tightly linked sets of QTN within a QTL, such as the *ASI2* alleles likely to be linked to other alleles of opposite phenotypic effect. In such cases the highly selected pool can identify these complex situations by enriching for rare recombinant haplotypes in the region (69). The identification of overlapping QTL regions among progeny from different tetraploid hybrids allowed us to focus on the genes that are more likely to be responsible for the phenotypic variation. Such an approach can also identify alleles which exert a background-independent effect.

With the sterility of hybrids overcome, we have shown that the hybrid situation is even more complex than the complex trait analysis within a species.

Firstly, the type of mitochondria inherited affect the QTL landscape. We were able to compare QTL regions mapped in diploid hybrid progeny derived from tetraploid parental lines with different mitochondria. Interestingly, the majority of QTLs detected were exclusive to a specific mitotype, and only a small number of QTL regions were in common among the segregants from tetraploid hybrids with different mitochondria. Although the extent of the mitochondrial–nuclear epistasis cannot be easily extrapolated from these data, these results reinforce the idea that genetic interactions between the mitochondrial and nuclear genome are important. Studies on interspecies yeast hybrids in the laboratory have shown a correlation between the origin of the mitochondrial genome and the higher stability of one of the nuclear genomes (74, 75). Moreover, several mitochondrial–nuclear incompatibilities leading to respiratory deficiencies have been identified in yeast hybrids (50), and some are associated with the splicing of mitochondrial intron cox1I3β (76). The incompatibility of the *S. uvarum* mitochondrion with the *S. cerevisiae* nucleus was reinforced by the transplacement of mitochondria isolated from *S. uvarum* (77, 78). A recent study on *S.cerevisiae*/*S. uvarum* hybrids has also shown that selective mitochondria retention is influenced by its contribution to hybrid fitness in different environments, and the type of mitochondria inherited affects the nuclear transcription

at alleles level (18). In yeast, mitochondrial–nuclear epistasis also contributes to coadaptation to changing environmental conditions (53, 54, 79, 80). These mitochondrial–nuclear interactions are examples of the growing number of Bateson–Dobzhansky–Müller incompatibilities affecting the function of certain processes and fitness but are not directly leading to inviability/infertility, so-called speciation genes, which are quite rare in *Saccharomyces* (81). Mitochondrial–nuclear epistasis has been shown to affect phenotypes in several taxa. In insects, such as *Drosophila* and *Callosobruchus*, exchange of mitochondrial DNA variant has led to decreased metabolic rate (82) and shortened life span (83). In mice, it is known to affect cognition and respiratory functions (84, 85). Interactions between mitochondrial and nuclear genomes can result in cytoplasmic male sterility in plants (86) and impact aging and longevity in humans (87).

Secondly, and even more profound, new QTLs are generated in hybrids. This is allelic variation that has no phenotypic consequences in a parent species but has phenotypic consequences in the hybrid. RHA (25) on *ASI2*, *FUS3*, and *GIT1* was carried out to assess the fitness the Sc^{OS253} and Sc^{OS104} alleles in the interspecific tetraploid hybrid ($Sc^{OS253}/Sk^{OS575}/Sc^{OS104}/Sk^{IFO1802}$) and in the *S. cerevisiae* intraspecific diploid hybrid (Sc^{OS104}/Sc^{OS253}), and we showed that these QTLs are exclusive to interspecies hybrid background. The potential, therefore, for exploiting natural genetic variation in developing new hybrids is greater than expected and bodes well for future advances in yeast breeding for improvement.

Materials and Methods

Strains, Growth Conditions, and Sporulation. The complete list of diploid strains used in this study is shown in Dataset S5. These strains were chosen for the creation of de novo interspecies and intraspecies hybrids. Yeast strains were routinely cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose, Formedium). To select for the drug resistance markers, YPD medium was supplemented either with 300 μg/mL geneticin, 300 μg/mL hygromycin B, 100 μg/mL nourseothricin, or 10 μg/mL bleomycin.

Construction of Genetically Stable Haploid Strains. All the haploid strains used in this study are listed in *SI Appendix*, Table S13. Genetically stable haploid *S. cerevisiae* strains used to make the hybrids were obtained from the Louis laboratory (88) and from derivatives of these (89). *S. jurei* haploid strain was constructed previously in Delneri laboratory (3). *S. uvarum*, *S. eubayanus*, and *S. kudriavzevii* haploid strains were engineered in this study, by deleting the HO gene. The plasmids and the primers used for the gene deletion and verification are listed in *SI Appendix*, Table S14 and Dataset S6, respectively. The methodological details for the generation of haploid strains are presented in *SI Appendix*, *Supplementary Methods*.

Generating Tetraploid Hybrids and Sporulation. Mass mating, sporulation, and tetrad dissection were conducted by following standard protocols (90). Two approaches to generate tetraploid hybrids were used (Fig. 1). The hybrid nature of the diploid spores was determined by species specific PCR (91), and the primers used are listed in Dataset S6. The detailed methodological procedure for hybrids construction is presented in *SI Appendix, Supplementary Methods*.

Multigenerational Advanced Intercross Lines. Tetraploids were subjected to sporulation, and tetrads were isolated for dissection to assess the spore viability and phenotypic variation and to prepare the spores for further rounds of mating. The methodological details for the construction of multigenerational hybrid lines are presented in *SI Appendix, Supplementary Methods*.

Analysis of Mitochondrial Origin in Hybrids. For each diploid mater, a petite version was generated by exposure to ethidium bromide (EtBr) (92). Isolates were seeded at an approximate density of 300 individuals per plate. A 3-μL drop of EtBr (10 mg/mL) was spotted onto the center of each plate. A ring around the spot formed where all cells were killed due to the toxic effects of EtBr. Surrounding this kill zone, a ring of petite colonies form. Loss of mitochondria enables petites to grow faster than colonies with functional mitochondria in the presence of EtBr. These individuals were confirmed as petites by their inability to grow on YPD plates containing ethanol and glycerol, nonfermentable carbon sources (93). The specific mitotype was identified by amplifying the *COX2* and *COX3* genes through colony PCR as described previously (18). The primers used for the amplification of these genes are listed in Dataset S6.

Phenotypic Assays. A high-throughput spot assay was performed using Singer ROTOR HDA robot (Singer Instruments, United Kingdom) as mentioned previously (2). The fitness of ~384 hybrid spores was assessed at five different temperatures (i.e., 10, 16, 23, 30, and 37 °C) under different carbon sources at 30 °C (i.e., YPA + 10% & 15% maltose, YPA + 10% & 15% fructose, YPA + 10% & 15% sucrose, YPA + 10% & 15% galactose, and YPA + 30% & 35% glucose) and under different environmental stressors at 30 °C (i.e., YPAD + 6% & 10% ethanol, YPAD + 0.3% & 0.5% acetic acid, YPAD + 4 mM hydrogen peroxide, YPAD + 50 mM levulinic acid, and YPA + 10% & 15% glycerol).

Fitness analysis was done following two different strategies, using either Phenosuite software (Singer Instruments) or the PHENOS platform (94). Details of the phenotypic analysis are given in *SI Appendix, Supplementary Methods*.

Sequencing, Mapping, and Variant Calling. Each parental genome in the hybrid was sequenced previously (2) or in this study at the Earlham Institute or at the Genomic Technologies Core Facility of the University of Manchester. Two different pooling strategies were followed for Multipool and Pooled selection, respectively. In strategy one, from each selective media plate, the top 20 performing F12 individuals, with the highest fitness, and the 20 lowest performing F12 individuals were picked for pooling. In second strategy, a pool of 1×10^8 F12 cells were seeded onto each selection condition as well as the YPD control. Selection conditions were prepared so that only the top 0.1 to 1% of the pool would be capable of growth.

The sequencing was done to 100 to 120× coverage on the Illumina platform.

Paired-end raw Illumina sequence reads were quality checked through FastQC 0.11.5 (95) and trimmed through Trimmomatic 0.36 (96). The complete variant calling pipeline is displayed in *SI Appendix*, Fig. S4 and described in *SI Appendix*.

QTL Mapping. Two different strategies for detection of QTLs have been followed for the Multipool and for Pooled selection, respectively. The detailed methodological pipeline for both approaches is given in *SI Appendix, Supplementary Methods*. QTL regions associated with the phenotype were identified by analyzing the changes in frequencies of SNP alleles across the genomes. The QTL intervals, gene content, and LOD scores are included in Dataset S2.

GO and SIFT Analysis. GO terms were determined using the GO Term Finder tool of Saccharomyces Genome Database with the Bonferroni correction for multiple hypothesis and a *P* value cutoff of 0.01.

Potential causal genes were analyzed with the SIFT algorithm to assess whether amino acid variants were predicted to influence the protein function. SIFT analysis were conducted using data from Bergström et al. (44) on the *S. cerevisiae* strains OS3, OS104, and OS253 and the tool mutfunc (65).

Validation of Candidate Genes through RHA. The candidate genes selected for RHA were chosen based on their LOD score and on GO studies carried out with YeastMine (97) and Funspec (98) to prioritize terms connected to the phenotypic trait tested.

We performed RHA (25) on the selected genes *ASI2*, *FUS3*, and *GIT1*. PCR-mediated deletion of the target genes was performed in the engineered *S. cerevisiae*/*S. kudriavzevii* hybrid (Sc^{OS253}/Sk^{OS575} and $Sc^{OS104}/Sk^{IF01802}$) and on *S. cerevisiae* haploid strains (Sc^{OS253} and Sc^{OS104}) to delete the *S. cerevisiae* allele in each of them. Tetraploid interspecific reciprocal hemizygotes were constructed by mating the two diploid *S. cerevisiae*/*S. kudriavzevii* hybrids (Fig. 5*A*). Diploid intraspecific reciprocal hemizygotes were constructed by mating the two *S. cerevisiae* strains (Fig. 5*E*). The strains constructed for RHA are listed in *SI Appendix*, Table S15. The fitness of the *S. cerevisiae* allelic variants of *FUS3*, *GIT1*, and *ASI2* was assessed both in the hemizygous *S. cerevisiae*/*S. kudriavzevii* tetraploids and in the hemizygous *S. cerevisiae* diploids. The methodological details for the construction of the strains and fitness analysis are presented in the *SI Appendix, Supplementary Methods*.

Data Availability. Sequencing data have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/view/PRJEB44105) (99). All other study data are included in the article and/or supporting information.

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