**SUPPLEMENTARY FIGURE CAPTIONS**

**Supplementary Figure 1. Making barcoded hemizygotes in a yeast hybrid background for RH-seq.** (A) A pool of random N20 barcodes (colors), each flanked by universal priming sites (U1 and U2), was used as input into a PCR with primers containing recognition sites for the BbsI type IIS restriction enzyme. (B) In a plasmid harboring an un-barcoded piggyBac transposon (gray rectangle) (the kanamycin resistance cassette, kanR, flanked by left and right transposon arms) and transposase (teal rectangle) (Weiss et al. 2018), a 42 nucleotide stuffer sequence, consisting of two BbsI restriction enzyme sites flanking a NotI restriction enzyme site and custom overhang sequences (Lee et al. 2015), replaced 42 nucleotides in the right arm of the transposon. (C) BbsI digestion of the barcodes and stuffer-containing plasmid, followed by stuffer loss and ligation of a barcode into each plasmid, yielded a pool of barcoded plasmids. (D) Transformation of the barcoded transposase plasmid into *S. cerevisiae* x *S. paradoxus* hybrids, followed by transposition and plasmid loss, yielded a pool of marked transposon hemizygote insertion genotypes in the hybrid background.

**Supplementary Figure 2. Modifying yeast piggyBac to test barcode insertion positions and transposase optimization, and effects of barcode insertion positions and transposase sequence on yeast piggyBac transposition efficiency.** (A) Left, a plasmid from (Weiss et al. 2018) containing the unbarcoded piggyBac transposon (gray) and transposase (teal) was modified to eliminate three BbsI restriction enzyme sites, and used as a backbone for further modifications. Right, test plasmids were mutated at transposase sites designed to optimize codons and increase activity (Yusa et al. 2011). Bottom, test plasmids were modified to incorporate into the transposon a single 20 nucleotide barcode flanked by universal priming regions and custom two-nucleotide overhang sequences (blue squares), either by insertion between the 3’-most end of the left arm and 5’ end of the TEF promoter of the kanamycin cassette (bottom left) or replacing endogenous nucleotides inside the right arm of the transposon (bottom right). Pink rectangles indicate transposase binding sites from (Morellet et al. 2018). (B)Each pair of boxes reports transposition test results from a plasmid schematized in (A) in the *S. cerevisiae* x *S. paradoxus* F1 hybrid, with transformation at the indicated temperature. For a given box, the thick black line reports the median; the box extent report quartiles; whiskers report outliers.

**Supplementary Figure 3. Gene coverage and read depth in thermotolerance Bar-seq.** (A) The *x*-axis reports the number of inferred hemizygote clones in a given gene (corresponding to transposon insertion mutants) whose abundance was detectable in Bar-seq (see Figure 1B), and each bar height reports the number of genes with the number of detectable hemizygotes on the *x*, for the indicated species’ allele in the diploid hybrid. The dotted red line indicates the cutoff used in our quality control pipeline for tests of allelic impact on thermotolerance, whereby only genes with greater than three inserts for an allele in the Bar-seq counts were considered. (B) The *x*-axis reports the total number of Bar-seq reads, for a given inferred hemizygote clone in the indicated species’ allele, in competition cultures grown at 28°C; each bar height reports the number of inferred hemizygote clones with the Bar-seq abundance on the *x*. (C) Data are as in (B) except that competitions at 37°C were analyzed.

**Supplementary Figure 4. Impact on high-temperature growth of allelic variation, in barcoded RH-seq, at genes from a previous thermotolerance screen.** (A) Each row reports the allelic effect, the thermotolerance conferred by disruption of the *S. cerevisiae* allele, relative to the analogous quantity for the *S. paradoxus* allele, as measured in barcoded RH-seq, of a gene at which allelic variation was previously reported to impact thermotolerance (Weiss et al. 2018). Genes marked with asterisks were significant at *p* < 0.05, after quality control for noise and number of inserts and multiple-hypothesis correction. (B) The *x*-axis reports allelic effect for a given gene as in (A); the *y*-axis reports the proportion of genes with the allelic effect on the *x*, with the blue trace showing the distribution across all genes with barcoded RH-seq data, as a kernel density estimate. Red dotted vertical lines represent genes from (A).

**Supplementary Figure 5**. **Accelerated evolution of thermotolerance loci.** Shown are results of analyses of branch length of top hit genes from barcoded RH-seq mapping of thermotolerance, as inferred from gene trees and normalized for gene length. Each vertical bar reports inferred branch length, along the *S. cerevisiae* lineage, of the indicated RH-seq hit gene. Horizontal lines report median branch lengths across the indicated gene sets. A resampling test for long branches on the *S. cerevisiae* lineage among top RH-seq hits revealed significant evidence for enrichment (*p* = 0.0465) but not when *TAF2* and *BUL1* were eliminated (*p* = 0.1574), attesting to the particularly strong inference of accelerated evolution in the latter two genes.

**Supplemental Literature Cited**

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