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Genetic, genomic, and molecular tools for studying the protoploid yeast, *L. waltii*

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Abstract

Sequencing of the yeast *Kluyveromyces waltii* (recently renamed *Lachancea waltii*) provided evidence of a whole genome duplication event in the lineage leading to the well-studied *Saccharomyces cerevisiae*. While comparative genomic analyses of these yeasts have proven to be extremely instructive in modeling the loss or maintenance of gene duplicates, experimental tests of the ramifications following such genome alterations remain difficult. To transform *L. waltii* from an organism of the computational comparative genomic literature into an organism of the functional comparative genomic literature, we have developed genetic, molecular and genomic tools for working with *L. waltii*. In particular, we have characterized basic properties of *L. waltii* (growth, ploidy, molecular karyotype, mating type and the sexual cycle), developed transformation, cell cycle arrest and synchronization protocols, and have created centromeric and non-centromeric vectors as well as a genome browser for *L. waltii*. We hope that these tools will be used by the community to follow up on the ideas generated by sequence data and lead to a greater understanding of eukaryotic biology and genome evolution.

Keywords

mating type; ploidy; transformation; genome browser; karyotype; cell cycle

Introduction

The advent of high-throughput genome sequencing has opened the gateway to greater understanding and increased experimental exploration of new organisms. For example, sequencing of the yeasts has expanded our knowledge of population heterogeneity (Diezmann and Dietrich, 2009; Liti *et al.*, 2009; Schacherer *et al.*, 2009), industrially relevant species (Borneman *et al.*, 2008; De Schutter *et al.*, 2009; Jeffries *et al.*, 2007; Nakao *et al.*, 2009; Novo *et al.*, 2009), pathogenicity (Butler *et al.*, 2009) and genome evolution (Dietrich *et al.*, 2004; Dujon *et al.*, 2004; Scannell *et al.*, 2007; Souciet *et al.*, 2009). In particular to this last point, sequencing of the yeast *Kluyveromyces waltii* (Kellis *et al.*, 2004), hereafter referred to as *Lachancea waltii* (Kurtzman, 2003), confirmed the

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Supporting Information

Supporting information may be found in the online version of this article.

occurrence of a whole genome duplication (WGD) event in the yeast lineage leading to *Saccharomyces cerevisiae*.

L. waltii is a protoploid or preWGD budding yeast species, a yeast descended from a lineage that did not undergo the whole genome duplication. *L. waltii* — type strain CBS 6430 (NRRL Y-8285) — was first isolated in 1974 in Japan from *Ilex integra* (the Mochi Tree; Kodama and Kyono, 1974) and was placed in the *Kluyveromyces* genus, which originally included yeasts of the *Saccharomyces* complex that produce evanescent asci (van der Walt, 1965). However, when sequences for the rDNA, elongation factor EF-1 α , CoxII and other loci became available, *L. waltii* was reassigned to the new genus *Lachancea* (Kurtzman, 2003).

Analysis of the ~11 Mb *L. waltii* genome by Kellis *et al.* (2004) revealed the general pattern that for each region in *L. waltii* there are two syntenic regions in *S. cerevisiae*, a pattern that is consistent with a whole genome duplication event followed by massive gene loss in the *S. cerevisiae* lineage. *L. waltii* therefore is of value both in studying the biology of pre-WGD yeasts and in comparative genomic analysis of post-WGD yeasts. Accordingly, the publication of the *L. waltii* genome was met with much discussion not only on the utility but also on the limitations of working with *L. waltii*. In one commentary, Wolfe (2004) wrote

The publication of the ... [*L.*] *waltii* genome [sequence] opens the door to detailed analysis of how *S. cerevisiae* evolved. Although complete genome sequences are invaluable, real experiments will also be necessary to give us a better understanding of the biology of pre-duplication species ... [*L. waltii*] is not ideal in this regard [for it] is almost completely uncharacterised and no laboratory tools have been developed for it.

Indeed, at the time of publication of its genome sequence, *L. waltii* was very poorly developed for laboratory study. Some basic growth preferences had been characterized (Barnett *et al.*, 2000; Bitzilekis and Barnett, 1997; Kurtzman and Fell, 1998) and a partial karyotype had been reported (Belloch *et al.*, 1998). Transformation of *L. waltii* had been accomplished using a protoplast-based procedure and a plasmid containing the autonomously replicating sequence (ARS) of the endogenous *L. waltii* 2 μ plasmid (Chen *et al.*, 1992). Shortly after the *L. waltii* genome sequence became available, it was added to the Yeast Gene Order Browser (YGOB) (Byrne and Wolfe, 2005). YGOB, however, displays the *L. waltii* genome in an unassembled, contig-based form (Byrne and Wolfe, 2005), which limits its use.

Over the more than 80-year history of *S. cerevisiae* research, this simple budding yeast has been transformed into the pre-eminent model eukaryote for studies in biochemistry, molecular biology, cell biology and genetics. Although we cannot hope to develop *L. waltii* to the extent that *S. cerevisiae* has been developed for research, we have greatly improved the tractability of working with *L. waltii*. Here we describe our successes in understanding optimal growth conditions, drug sensitivities, cell cycle progression, ploidy, karyotypes and mating for *L. waltii*. Also, we provide to the community protocols for high efficiency transformation, cell cycle arrest and tools such as *L. waltii* cloning vectors, two auxotrophic strains and an *L. waltii* genome browser based on an assembled genome.

Methods

Growth conditions and media

Yeast cells were cultured in various media as follows. Rich medium (YEP) consisted of yeast extract (10 g/L), peptone (20 g/L) and either dextrose (2% w/vol; YEPD) or glycerol (3% vol/vol; YEP + glycerol). YM medium contained yeast extract (3 g/L), malt extract (3

g/L), peptone (5 g/L) and dextrose (10 g/L). Liquid synthetic complete medium (YC) consisted of ammonium sulfate (5 g/L), yeast nitrogen base (without either amino acids or ammonium sulfate; 1.45 g/L), 20 g/L succinic acid, pH adjusted to 5.8 using NaOH, and supplemented with leucine, isoleucine and tryptophan (77 mg/L); adenine, arginine, uracil, histidine and methionine (19 mg/L); aspartic acid and glutamic acid (96 mg/L); threonine (192 mg/L); valine (144 mg/L); phenylalanine (48 mg/L); lysine and tyrosine (58 mg/L); serine (384 mg/L); and either dextrose (2% w/vol; YC + glucose) or glycerol (3% vol/vol; YC + glycerol). Synthetic complete plates (YC) contained ammonium sulfate (5 g/L), yeast nitrogen base (without either amino acids or ammonium sulfate; 1.7 g/L), Difco agar (20 g/L), and supplements as above except where dropouts are noted. Nitrogen starvation medium (-N) consisted of yeast nitrogen base (without either amino acids or ammonium sulfate; 1.61 g/L), succinic acid (11.1 g/L), sodium hydroxide (6.66 g/L) and ammonium sulfate (0.8 g/L). Dilute YM medium (DYM) contained yeast extract (0.3 g/L), malt extract (0.3 g/L), peptone (0.5 g/L), Difco agar (20 g/L) and dextrose (1 g/L). All *L. waltii* cultures were incubated at 23 °C unless otherwise noted.

Flow cytometry

Cells were prepared for flow cytometry as described previously (Nash *et al.*, 1988) with some minor modifications. Briefly, 0.5 ml cells were collected and 1 ml of 100% ethanol was added. Cells were pelleted and then resuspended in 0.5 ml 50 mM sodium citrate (pH 7.4) and sonicated as done routinely for *S. cerevisiae*. A 0.5 ml aliquot of sodium citrate plus RNase A (0.25 mg/ml) was added and cells were incubated at 50 °C for 1 h. Next, 50 µL Proteinase K (20 mg/ml) was added and cells were incubated for an additional hour at 50 °C. Sytox Green (Molecular Probes) was added to a final concentration of 1 µM. Flow cytometry was performed on a BD FACScan and data were analyzed using BD FACStation (BD Biosciences).

Cell cycle manipulation

Nocodazole (Sigma-Aldrich) was added to YEPD at a final concentration of 15 µg/ml. Hydroxyurea (Sigma-Aldrich) was added to YEPD at a final concentration of 100 mM. Nitrogen starvation was executed by harvesting a logarithmically growing culture of *L. waltii* cells, resuspending the cells at an OD_{660nm} of 0.1 in nitrogen starvation medium, and then incubating at 23 °C for 20 h. For the halo assay, synthetic *S. cerevisiae* α -factor peptide (United Biochemical Research) and *L. waltii* α -factor peptide (Genscript) were spotted (200 µg per filter disk), with and without the addition of 50 µg Pronase (Calbiochem). *L. waltii* response to *S. cerevisiae* α -factor was tested in liquid culture using concentrations of 15 and 75 µM in YEPD.

DNA isolation

DNA was isolated from *L. waltii* following either the 'smash-and-grab' protocol of Rose *et al.* (1990) with modifications described at <http://fangman-brewer.genetics.washington.edu/smash-n-grab.html> or using the method described by Huberman *et al.* (1987) with the modifications described at http://fangman-brewer.genetics.washington.edu/DNA_prep.html.

Southern blotting and probes

L. waltii DNA for Southern blot analysis was prepared using the smash-and-grab protocol from either 1.5 ml of log phase cells in rich medium, or from a single colony resuspended directly in the smash-and-grab lysis solution. One-tenth of the DNA so prepared was digested for Southern blot analysis.

Probes for Southern blotting were PCR amplified from *L. waltii* DNA and gel purified. The mating type locus was probed for a portion of the gene *KLLA0D00396g*, which is common to both mating types, using primers MAT1-1 and MAT1-2. The probe for the *L. waltii* rDNA locus was amplified using the primers Lw18S-1 and Lw18S-2, which hybridize to the 18S rDNA sequence (accession number X89527). The probe to confirm the *ura3* deletion was amplified using the primers LwURA3-3 and LwURA3-4. The *his3* deletion mutation was confirmed by random primed labeling of the plasmid pGEM-*Lwhis3Δ::KanMX* (see below). All primer sequences can be found in Supporting Table S1 (see Supporting Information).

Plasmid construction

L. waltii vectors containing a selectable marker, a centromere, and an origin of replication were constructed using pUC18 as a backbone. All primer sequences can be found in Supporting Table S1. Into pUC18 we first cloned KanMX from pUG6 using primer pair KanMX_F and KanMX_R. These primers enable directional cloning into the EcoO109I–AatII region on pUC18. The primers were designed so that the region of KanMX amplified from pUG6 would include the MluI site from pUG6. *L. waltii* CEN7 was then cloned into this MluI site on our pUC18:KanMX plasmid using primers CEN7_F and CEN7_R. Cloning of KanMX and CEN7 was confirmed using primers pUC18:KanMX_Seq_F and pUC18:KanMX_Seq_R. A fragment that contained the ARS was obtained from the endogenous 2 μ plasmid pKW1. This fragment, which includes and lies between the sequences (5'-ATTCCTTACCATTTTCGTCCCTCATGATG-3') and (5'-GTGCGTTCTTAAAGCTGAGTAGGCACTTCA-3'), was blunt-end cloned into the SmaI site within the multiple-cloning site of the pUC18 backbone. The sequence of this ARS was confirmed using primers Vector_Insert_F and Vector_Insert_R.

The *ura3* deletion construct was made by PCR amplifying a 2.1 kb fragment that includes the *L. waltii* URA3 gene, currently annotated as gene 16 109, using primers LwURA3-3 and LwURA3-4. A 1.3 kb EcoRI–HindIII fragment containing URA3 was then excised from the 2.1 kb PCR product and gel purified before cloning into pUC18 to generate pUC18–*LwURA3*. A 428 bp deletion within the URA3 ORF was created *in vitro* by digesting pUC18–*LwURA3* with EcoRV and XbaI, filling in the XbaI site using *exo*[−] Klenow (New England Biolabs) to generate a blunt end, and then recircularizing the fragment to create pUC18–*Lwura3Δ*. The *ura3Δ* cassette was then excised from pUC18–*Lwura3Δ* and introduced into *L. waltii* cells using the chemical transformation protocol described here; the cells were plated on medium containing 5-fluoroorotic acid (5-FOA; 1 g/L) to select for *ura*[−] transformants.

The *his3* deletion construct was made by PCR amplifying a 1.7 kb fragment that includes the *L. waltii* HIS3 gene, currently annotated as gene 8528, using primers LwHIS3-1 and LwHIS3-2 and Easy-A Polymerase (Stratagene). The *HIS3* fragment was then cloned into the pGEM-T Easy vector (Promega) by virtue of the A-overhangs to create plasmid pGEM–*LwHIS3*. Next, a 300 bp deletion was created within the *HIS3* open reading frame by PCR amplifying the pGEM–*LwHIS3* plasmid using primers His3delta-1 and His3delta-2, which contain an artificial BamHI site, and Pfu Turbo polymerase (Promega). Finally, the KanMX gene was inserted at the site of the *his3* deletion to create plasmid pGEM–*Lwhis3Δ::KanMX*. The *his3Δ::KanMX* construct was then excised and integrated into the *L. waltii* genome using the electroporation protocol described here. Transformants were selected on YEPD plates containing G418 and integration at the *HIS3* locus was confirmed by Southern blotting.

Transformation

Chemical transformation of *L. waltii* was accomplished by first creating frozen competent cells and then transforming thawed cells in the presence of lithium acetate (LiAc) and dithiothreitol (DTT). To create frozen competent cells, a 50 ml culture of *L. waltii* was grown at 23 or 30 °C in YEP + 3% glycerol for at least 3 generations to a culture density of $OD_{660\text{ nm}} = 0.5\text{--}0.7$ ($\sim 7 \times 10^6$ cells/ml). Cells were then pelleted for 5–10 min at less than 1000g, resuspended in 1 ml TE–LiAc (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.1 M LiAc pH 7.5), and pelleted again for 15 s at 16,000g. The washed cells were resuspended in 50 ml of TE–LiAc and incubated with shaking for 1–1.5 h at 23 or 30 °C. For storage, competent cells were aliquoted into microfuge tubes, which were wrapped in multiple layers of Kimwipes to promote slow cooling and stored in cardboard containers at –80 °C.

On the day of transformation, 0.5–2.0 µg of DNA to be transformed was premixed with 5 µl of freshly denatured herring sperm DNA (10 mg/ml). A 100 µl aliquot of thawed competent cells were added to the DNA followed by the addition of 700 µl of PEG–TE–LiAc (40% PEG 3350, TE–LiAc) and 0.815 µl of 1 M DTT. Cell suspensions were mixed by gentle vortexing, incubated with shaking at 23–30 °C for 30 min, and heat shocked at 42 °C for 15 min. Cells were recovered by centrifugation for 15 s at 3000–5000g. The use of KanMX as the transformation marker requires a period of growth in non-selective culture medium before exposure to the selective agent, G418. Accordingly, cell pellets were resuspended in 150–200 µl of –N medium and plated onto YEPD plates and allowed to grow for 8–20 h before replica-plating to YEPD + G418 plates (200 µg/ml).

Electroporation was carried out as described previously for *S. cerevisiae* (Ausubel, 1997) with minor modifications. A 50 ml culture of *L. waltii* was harvested at an OD of 1.0 by centrifugation at 4 °C and 4000g. Cells were resuspended in 8 ml water, followed by the addition of 1 ml of 10× TE, pH 7.5, and 1 ml 1 M lithium acetate with gentle rotation at 30 °C for 45 min. Next, 0.25 ml of 1 M DTT was added, followed by an additional 15 min incubation under the same conditions. Cells were washed three times: in 50 ml ice-cold water, in 25 ml ice-cold water, and in 2.5 ml ice-cold 1 M sorbitol. Finally, cells were resuspended in 50 µl ice-cold 1 M sorbitol for a final volume of about 200 µl of cell suspension. For each electroporation, 50 µl of cells were transferred to ice-cold 0.2 cm electroporation cuvettes (Fisher), and 1 µg DNA (volume <5 µl) was added. Electroporation was performed using a GenePulser (Biorad) with pulse conditions of 1.5 kV, 25 F, 200 Ω. After the pulse, 1 ml of ice-cold 1 M sorbitol was added, cells were transferred to prechilled eppendorf tubes and kept on ice until plating on selective medium.

qPCR

The copy number of the ARS plasmid relative to the CEN–ARS plasmid was assayed by quantitative real-time PCR (qPCR) using the LightCycler 480 platform and the SYBR Green I Master kit (Roche). Cycling conditions were 5 min at 95 °C followed by 40 cycles consisting of 10 s at 95 °C, 10 s at 56 °C and 15 s at 72 °C after which fluorescence from SYBR Green was read. Melting curve analysis was used to determine whether each qPCR reaction generated a unique product. Cycle threshold (C_t) values were calculated using the absolute quantification/second derivative max function available on the LightCycler 480 software. All reactions were run in triplicate and the mean C_t values were used in subsequent calculations. The copy number of the ARS plasmid relative to that of the CEN–ARS plasmid was determined by computing the $\Delta\Delta C_t$ value (Livak and Schmittgen, 2001) for the plasmid-specific products, β -lactamase and KanMX. As controls, the relative copy numbers of *L. waltii* chromosomal genes 1109 (*ACT1*) and 14 267 (*TUB1*) were calculated for DNA samples from yeast with the ARS plasmid, the CEN–ARS plasmid, or no plasmid.

A primer set for *CEN7* was used to determine the copy number of the CEN–ARS plasmid. All qPCR primers are described in Supporting Table S1.

CHEF gel electrophoresis

L. waltii cells were embedded in plugs of 0.8% agarose and prepared for CHEF (Contour-clamped Homogenous Electric Field) gel electrophoresis as described by Iadonato and Girke (1996), with modifications as noted at <http://fangman-brewer.genetics.washington.edu/plug.html>. CHEF gel electrophoresis was performed in a CHEF-DR II system (Biorad) in 0.5× TBE at 14 °C using a three-step protocol of ramp times: 600–1200 s, 110 V, 49 h; 120–400 s, 128 V, 48 h; and 60–120 s, 160 V, 24 h. After electrophoresis, the gel was stained with ethidium bromide, photographed and blotted for Southern hybridization using probes described in Supporting Table S1. Plugs of *S. cerevisiae* cells provided the size standards for electrophoresis.

Browser

The *L. waltii* genome sequence was assembled as previously described (Di Rienzi *et al.*, 2009). Briefly, ordered contigs described by Kellis *et al.* (2004) were joined by an ‘N’ to represent a gap. Open reading frame (ORF) and centromere calls were derived from those made by Kellis *et al.* (2004) and tRNA predictions were taken from Di Rienzi *et al.* (2009). *MATa* and *MATα* idiomorphs of chromosome V were created by filling the gap on chromosome V at 279 301 bp. The sequences of the *MATa* and *MATα* mating loci were taken from GenBank accessions AM748704.1 and AM748703.1, respectively. Kellis or Wolfe *S. cerevisiae* homologs and the yeast Ancestor homolog matches to each *L. waltii* gene were gathered from Kellis *et al.* (2004) or Byrne and Wolfe (2005) and Gordon *et al.* (2009), respectively. *S. cerevisiae* and Ancestor genes were colored to represent the chromosome to which they belong in their native genome. Syntenic blocks between *S. cerevisiae* and *L. waltii* were identified by considering the locations of *S. cerevisiae* genes in their *S. cerevisiae* chromosomal order relative to their order as homologs in the *L. waltii* genome. To do so, each *S. cerevisiae* homolog to an *L. waltii* gene was again colored according to the *S. cerevisiae* chromosome to which the homolog belongs. Where adjacent *L. waltii* genes have homology to *S. cerevisiae* genes that are adjacent in their native *S. cerevisiae* chromosome environment, a syntenic block was created to include both *S. cerevisiae* homologs. These blocks were connected when the genes/homologs bounding the blocks are *L. waltii* genes within 20 genes of each other and the *S. cerevisiae* homologs are within 30 genes of each other. These gene distances were selected because they minimized ambiguities in defining syntenic blocks. The *L. waltii* genome and all genomic features described above were formatted for display using Generic Model Organism Database software tool GBrowse (Stein *et al.*, 2002).

Results

Vegetative growth

We characterized the growth of *L. waltii* in various media and temperature conditions, the results of which are summarized in Table 1. *L. waltii* had a doubling time of approximately 2 h at 23 °C in standard *S. cerevisiae* rich or complete media supplemented with 2% glucose. Much slower growth rates were observed when 3% glycerol was used as the carbon source. However, because *L. waltii* grows as well on rich or complete media with no defined carbon source as it does on the same media with glycerol, it is unclear to what extent *L. waltii* utilizes glycerol, and instead may be using other compounds for carbon and energy sources.

To determine the optimal growth temperature for *L. waltii*, cells were spotted onto YEPD plates and incubated at 15, 20, 23, 30, 35 or 37 °C for 3 days (Fig. 1). *L. waltii* was capable of growing at all temperatures except 37 °C, with colonies growing the most quickly and robustly at 30 °C. While little growth was observed at 15 °C, colony growth resumed when the plates were returned to room temperature. This conclusion is corroborated by our observation that *L. waltii* grows, albeit very, very slowly, at 4 °C. In contrast, growth did not resume for cells initially plated at 37 °C, and colonies growing at 35 °C displayed heterogeneity in colony size, suggesting that there is a growth defect at this temperature. We conclude that *L. waltii* cells are naturally temperature sensitive at 37 °C.

To determine the ploidy and to characterize the cell cycle stages of *L. waltii*, we compared the total DNA content of *L. waltii* (estimated haploid genome size of approximately 11 Mb; Kellis *et al.*, 2004) with that of *S. cerevisiae* (haploid genome size of approximately 12.1 Mb; SGD, 2010) using flow cytometry. Asynchronously growing *L. waltii* has a budding index of 75%, and flow cytometry shows a single population of cells with DNA content similar to the 2C content of haploid *S. cerevisiae* (compare Fig. 2A–D). Nitrogen starvation of *L. waltii* resulted in an unbudded cell population with a DNA content similar to the 1C content of haploid *S. cerevisiae* (compare Figure 2B with C). Together, these data suggest that *L. waltii* prefers vegetative growth as a haploid and spends the majority of its cell cycle in G2.

L. waltii is sensitive to several drugs used routinely in work with *S. cerevisiae*. Cells are unable to form colonies on 5-FOA but, like *S. cerevisiae*, spontaneously give rise to rare resistant variants. *L. waltii* also appears to be sensitive to nocodazole as cultures exposed to nocodazole show an increase in large-budded cells, suggesting an accumulation at G2/M. Release from nocodazole treatment, however, did not produce a synchronous transit through the subsequent cell cycle (data not shown). Treatment with 100 mM hydroxyurea produces an accumulation of cells at G1/S as observed by flow cytometry (Fig. 2E). Release from hydroxyurea arrest results in a synchronous transit through S phase (Fig. 2F–H).

Mating type

Gordon and Wolfe identified the MAT locus in *L. waltii*, located approximately 250 kb from the silent cassettes, which are clustered together on the left arm of chromosome V, and submitted sequences for both the *MATa* and *MATα* idiomorphs (accession numbers AM748704 and AM748703, respectively; Fig. 3A). Given that the flow cytometry data presented above indicated that *L. waltii* grows vegetatively as a haploid, we expected each clonal population to be composed of a single mating type. Therefore we analyzed the MAT locus in six independent colonies (Fig. 3B, lanes a–f). Surprisingly, Southern blotting of the MAT locus using a probe that hybridizes immediately adjacent to the idiomorphic region revealed the presence of both *MATa* and *MATα* restriction fragments (Fig. 3B), although not at equal frequencies as would be expected for a culture of diploid cells. We interpret the presence of both mating types in clonally derived populations of cells to be the result of spontaneous but infrequent mating type switching among haploids. As *L. waltii* lacks both a recognizable *HO* gene and *HO* recognition sequences in the mating type cassettes (Fabre *et al.*, 2005), switching probably occurs via an alternative mechanism. Neither flow cytometry nor Southern blots on numerous independent colonies and liquid cultures showed evidence of a significant population of diploids under rich media conditions.

Given our observation that *MATa* and *MATα* cells could be cultured together without mating, we asked whether vegetatively growing *L. waltii* would respond to the presence of mating pheromone. The *S. cerevisiae* *MFalpha1* and *MFalpha2* genes have a common ortholog in the *L. waltii* genome, currently annotated as gene 11 171, which encodes the presumptive precursor of the α -factor mating pheromone. Gene 11 171 encodes five perfect

repeats of a 13 amino acid peptide that bears a high degree of identity to *S. cerevisiae* α -factor (Fig. 4A). In addition, each peptide is bound by conserved proteolytic processing sites. Taken together, we hypothesized that this 13 amino acid peptide is the functional *L. waltii* α -factor. To test whether *L. waltii* would respond to α -factor mating pheromone, we spread a lawn of log phase cultures of predominantly *MATa* cells (as confirmed by Southern blotting) on YEPD plates, and placed paper disks containing synthetic α -factor from either *S. cerevisiae* or *L. waltii* on the lawn of cells. *S. cerevisiae* *MATa* cells responded to both *S. cerevisiae* and *L. waltii* α -factor as indicated by a halo of growth inhibition surrounding the paper disk. This response was largely abolished by the prior addition of Pronase (Fig. 4B). In contrast, *L. waltii* showed no growth inhibition to either α -factor peptide (Fig. 4B), and did not produce the shmoo-like (Capp, 2002) cell morphology that is characteristic of *S. cerevisiae*'s response to α -factor (data not shown). In addition, log-phase liquid cultures of predominantly *MATa* *L. waltii* exposed to various concentrations of *S. cerevisiae* α -factor also showed no change in cell cycle profile or budding index (data not shown). The absence of mating pheromone response in rich medium could explain the lack of an appreciable diploid population in cultures of mixed mating type.

Transformation

Chen *et al.* (1992) succeeded in transforming *L. waltii* using a protoplast-based procedure and a recombinant replicative plasmid that uses the ARS of the endogenous *L. waltii* 2 μ plasmid (pKW1). In a similar manner, we optimized a chemical transformation protocol by assaying the efficiency of transformation of a pUC18-based plasmid bearing the pKW1 ARS (Fig. 5). As detailed in the Methods section, our transformation protocol is derived from the LiAc chemical transformation protocol developed by Gietz and Schiestl (2007). Cells are pregrown in medium with glycerol (glycerol improves the transformation efficiency by ~10 fold, similar to the finding with *S. cerevisiae*; B. Brewer, unpublished observation), incubated in LiAc, and frozen. During the transformation procedure, cells and plasmid DNA are incubated with PEG-TE-LiAc and DTT. To test the efficiency of our LiAc transformation of *L. waltii* with pUC18:KanMX : ARSpKW1, transformed cells were first plated onto rich medium and then replica-plated to selective G418 media. Our transformation protocol produced between 10^3 and 10^4 colonies per μ g of plasmid. As these colonies are counted following a replica-plating step, the transformation efficiency may be an underestimate. The ability to create transformation-competent *L. waltii* without having to rely on spheroplasting greatly simplifies molecular manipulations.

Colonies that contain the ARSpKW1 plasmid were variable in size and slightly nibbled (Fig. 5B), indicating that the plasmid is lost spontaneously during mitotic growth. To improve the stability of the *L. waltii* plasmid as a cloning vector, we inserted into pUC18:KanMX : ARSpKW1 the centromere of *L. waltii* chromosome 7 (*CEN7*). We could visually distinguish colonies arising from transformation with the ARS plasmid from colonies arising from transformation with the CEN-ARS plasmid: colonies from the CEN-ARS transformation were much more uniform in size and were smooth in outline (Fig. 5D). However, CEN-ARS transformed colonies were slower growing, suggestive of a lower copy number and therefore lower expression of KanMX.

To quantify the copy number of the ARS plasmid relative to the CEN-ARS plasmid, we performed quantitative real-time PCR (qPCR). ARS and CEN-ARS colonies growing on G418 and colonies lacking any plasmid growing on YEPD were scraped from plates. Total DNA was extracted following the method of Rose *et al.* (1990). Cycle threshold values were determined for amplicons of the plasmid genes β -lactamase and KanMX and for chromosomal genes 1109 (*ACT1*) and 14 267 (*TUB1*). By qPCR, there are between 7- and 9-fold more copies of the ARS plasmid per cell than there are copies of the CEN-ARS plasmid per cell (Supporting Table S2). To determine the copy number of CEN-ARS

plasmid, we compared the cycle threshold values for *CEN7* (the centromere on the CEN–ARS plasmid) among the no plasmid, ARS and CEN–ARS DNA samples. The resulting data indicate that *CEN7* is present at 1.3-fold more copies in the CEN–ARS sample than in either the ARS or no plasmid samples (Supporting Table S2). This result leads us to conclude that the CEN–ARS plasmid is present in a single copy but that a fraction of the cells in a colony do not contain the plasmid (data not shown).

Integrative Transformation in *L. waltii*

Using the high efficiency chemical transformation protocol described here and a PCR-generated KanMX cassette flanked by 60 bp of homology to the *URA3* locus, we were unable to recover 5-FOA resistant colonies, suggesting an integrative transformation efficiency of less than one transformant per microgram of linear DNA when homology is short. However, including larger regions of homology supported integrative transformation in *L. waltii*, allowing us to produce non-reverting mutations in both *URA3* and *HIS3*, *L. waltii* genes 16 109 and 8528, respectively. To generate the *ura3* knock-out allele we cloned the *URA3* locus with about 700 bp of flanking genomic sequence into pUC18 and created a large deletion spanning most of the *URA3* gene (Fig. 5E). With these longer regions of homology, we were able to recover 5-FOA resistant colonies. Southern blotting confirmed that the 5-FOA resistant colonies carried the expected deletion (data not shown). We similarly cloned a large fragment containing *HIS3*, generated a large internal deletion marked by the KanMX gene, and transformed the linear construct into *L. waltii* by electroporation (Fig. 5F). The majority of the resultant G418 resistant colonies were also auxotrophic for histidine, indicating that, when homology is large, integration occurs preferentially by homologous recombination.

Mating

The creation of two auxotrophic strains, fortuitously generated in backgrounds of opposite mating types, presented an opportunity to study mating and the potential for genetic analysis in *L. waltii*. Using conditions optimized for studies of mating response in *S. cerevisiae* (Jackson and Hartwell, 1990), we mixed our *MATa ura3* strain and our *MATa his3* strain in a ratio of 1 : 100, respectively, and co-cultured the two strains on a variety of media. After incubation for 6 days at 23 °C, we began visually screening for shmoos and tetrads (Fig. 6A) and plated the cells on YC-ura-his plates to test for complementation.

Indeed, we found that, when cells were allowed to mate on rich media (YEPD plates or 5% malt agar plates), we could recover rare diploid cells: G418-resistant colonies growing in the absence of histidine and uracil. We confirmed the ploidy of these diploids by flow cytometry (data not shown) and Southern blotting (Fig. 6B, lanes a and b). The diploid state appears to be stable through many generations of growth on solid and in liquid media.

We were able to induce sporulation on two types of media, both of which contained malt extract (Dilute YM and Malt Agar). We were able to recover G418-sensitive prototrophic colonies which, by flow cytometry (data not shown) and Southern blotting (Figure 6B, lanes c–f), represent haploid segregants.

It appears that mating between the genetically marked haploids and subsequent sporulation are relatively rare events, consistent with the lack of both an appreciable diploid population in cultures of mixed mating type and the lack of an α factor response in *MATa* cells. This behavior contrasts strongly with that of *S. cerevisiae*, where mating between cells of opposite mating type occurs 45%–90% of the time (Dutcher and Hartwell, 1983; Muller, 1985). Previous reports of sporulation in *L. waltii* state that mother–daughter mating generally precedes ascus formation (Barnett *et al.*, 2000; Kregervan Rij, 1979; Kurtzman

and Fell, 1998). The use of the complementing auxotrophic markers permitted us to select for mating between unrelated haploids. In addition, we were able to screen prototrophs for the presence or absence of the G418^R phenotype to distinguish between stable diploids and a specific class of haploid meiotic segregants. These stable haploid and diploid strains should facilitate further studies of mating, mating type switching, and the life cycle in *L. waltii*.

Karyotype

We performed CHEF gel electrophoresis on *L. waltii* and, using the conditions described here, we were able to achieve sufficient separation to see six chromosomal bands upon staining with ethidium bromide (Fig. 7). Southern blotting with unique chromosomal probes confirmed that each chromosome migrated at the expected mobility with the exception of chromosome VIII. Chromosome VIII ran at a size of approximately 2.5 Mb, more than a megabase larger than the size expected based on the available sequence. Chromosome VIII also had a somewhat ‘fuzzy’ appearance on the ethidium-stained gel, similar to what is seen for *S. cerevisiae* chromosome XII, suggesting that the rDNA locus resides on this chromosome. Indeed, a probe specific for the *L. waltii* rDNA repeats hybridized specifically to chromosome VIII. While each of the other *L. waltii* chromosomes was assembled into a single supercontig, chromosome VIII was left as three supercontigs. Although the *L. waltii* rDNA locus cannot be identified through synteny relationships with *S. cerevisiae*, it is most likely in one or both of the gaps on chromosome VIII that prevented final assembly of this chromosome.

For our CHEF gel analysis, we used the *L. waltii* type strain obtained from both the American (ATCC56500) and European (CBS6430) collections. During the course of our work, we observed one case of chromosome-size polymorphism (Fig. 7, lane 7a) — chromosome VII from a colony derived from CBS6430 ran at a size approximately 100 kb larger than expected. Despite this large karyotypic polymorphism, we observed no obvious growth difference between the two clones. Our observation is consistent with a recent report that showed significant intraspecies karyotypic polymorphism within the *Lachancea* genus (Naumova *et al.*, 2007), but is the first report of karyotypic variation within the same strain. We conclude that the readily observable changes in karyotype reflect the dynamic nature of the *L. waltii* genome.

Browser

To visualize the assembled *L. waltii* genome, we created a genome browser using the Generic Model Organism Database software tool GBrowse (Stein *et al.*, 2002). The *L. waltii* genome browser can be accessed at <http://fangman-brewer-gbrowse.gs.washington.edu/cgi-bin/gbrowse/Lwaltii/>. The browser displays the *L. waltii* genome we assembled previously by concatenating adjacent contigs (Di Rienzi *et al.*, 2009) and marks the positions of gaps. The original sequence of *L. waltii* placed a gap at the location of the mating locus on chromosome V. As previously mentioned, the sequences of the *MATa* and *MATα* were determined by Gordon and Wolfe (unpublished) and deposited in Gen-Bank. We have incorporated these sequences into our browser by creating two idiomorphs of chromosome V — chromosome V *MATa* and chromosome V *MATα* (Fig. 8A). On chromosome V *MATa*, the *MATa* locus is 4773 bp in length and includes two mating type specific genes, *MATa1* and *MATa2*, as well as *KLLA0D00396g*, a gene common to both mating types that was missed in the original assembly of *L. waltii*. The *MATα* locus on chromosome V *MATα* is 6001 bp in length and contains genes *MATα1*, *MATα2* and *KLLA0D00396g*. All three versions of chromosome V include the silent mating locus on the left arm of the chromosome.

The entire genome has been annotated to show the location of predicted ORFs, centromeres and tRNA genes (Fig. 8B). While the function of each *L. waltii* ORF is unknown, we can predict a probable function by considering the *S. cerevisiae* homologs. To this end, the browser displays the *S. cerevisiae* homologous genes annotated by Kellis *et al.* (2004) and Byrne and Wolfe (2005) as separate tracks. In addition, we have included on the browser a track showing the homologous gene predicted to be present in the hypothetical common ancestor of *S. cerevisiae* and *L. waltii* (Gordon *et al.*, 2009). For both the *S. cerevisiae* and Ancestor homologs, the homologous genes have been colored according to the *S. cerevisiae* or Ancestor chromosome from which they are derived. Finally, we have included on the browser a track illustrating syntenic blocks between *S. cerevisiae* and *L. waltii*. These syntenic blocks are in good agreement with those previously described (Kellis *et al.*, 2004).

Any region of the *L. waltii* genome can be retrieved by providing the search box on the browser with chromosomal coordinates in the format chr:start..end. Additionally, any ORF, centromere, tRNA, or homolog can be found by simply searching using the feature name. Clicking on any feature will bring up a separate window providing the sequence and further information if available. For *L. waltii* genes and their *S. cerevisiae* and Ancestor homologs, a link is provided to the gene or homolog in either the Yeast Gene Order Browser (<http://wolfe.gen.tcd.ie/ygob/>) or the Saccharomyces Genome Database (<http://www.yeastgenome.org/>).

Discussion

Experimental science has long relied upon tractable systems. While the model organism *S. cerevisiae* has been and continues to be the principal platform to understand the basic properties of eukaryotic cells, it is limited by its own biology. For example, until very recently (Drinneberg *et al.*, 2009), RNAi was thought to be absent in budding yeast and was studied predominantly in higher eukaryotes. Furthermore, evolutionary studies require multiple systems to perform comparative analysis. Therefore, advances in experimental science necessitate that new organisms be developed for experimental work. Here we report our efforts to characterize the basic properties of the protoploid budding yeast *L. waltii* and make available a set of genetic and genomic tools for working with this yeast.

These insights and resources for *L. waltii* will facilitate future studies. Already, computational analyses using *L. waltii* have uncovered intriguing biological phenomena that demand experimental follow-up. In comparing a panel of pre- and post-WGD yeasts including *L. waltii*, Wapinski *et al.* (2010) have uncovered that the WGD event led to subfunctionalization of transcription factor binding sites for ribosomal protein genes. Another group has found evidence that the WGD was followed by divergence in gene expression of respiration genes and that differential nucleosome occupancy around these genes may explain this divergence (Field *et al.*, 2009). Separately, *L. waltii* and nine other yeast species were used to uncover horizontally transferred genes (Rolland *et al.*, 2009). Additionally, our work here suggests that the mating system of *L. waltii* differs from that in *S. cerevisiae* and may help to elucidate the evolution of fungal mating systems.

In conclusion, we have laid a foundation for *L. waltii* to aid in addressing a variety of evolutionary, genetic, cellular, and molecular biology questions. While we were able to develop *L. waltii* through knowledge of *S. cerevisiae*, we can now use *L. waltii* in comparative works in turn to better understand *S. cerevisiae* and its evolutionary history.

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Figure 1.

Temperature tolerance and sensitivity of *L. waltii*. *L. waltii* cells were spotted as a three-fold dilution series onto YEPD plates and incubated at the indicated temperature for two days. Growth was observed at all temperatures except 37°C

Figure 2.

Flow cytometry of *L. waltii* showing haploid preference during mitotic growth. Flow cytometry of asynchronous *L. waltii* in rich medium (A) and under conditions of nitrogen starvation (B), with the majority of cells either budded or unbudded, respectively. Flow cytometry of asynchronous *S. cerevisiae* clearly differentiates between haploid (C) and diploid (D) cultures. (E) Treatment of *L. waltii* with 100 mM hydroxyurea produces an accumulation of cells in G1 or early S phase, followed by a synchronous progression through S phase after release (F–H)

Figure 3.

Evidence of rare mating type switching events in mitotically growing *L. waltii*. (A) The *a* and α idiomorphs of the *L. waltii* MAT locus. The gray bar indicates the fragment labeled for Southern blotting. (B) Southern blots of the *L. waltii* MAT locus showing that, for six independent clones each derived from a single cell (a–f), both mating types can be detected, although in variable proportions

Figure 4.

Failure of *L. waltii* to respond to α -factor. (A) BLAST alignment of the *S. cerevisiae* α -factor peptide with the homologous *L. waltii* peptide. Amino acid identities are shaded. (B) Test of response to α -factor. Filter disks spotted with 200 μ g of *S. cerevisiae* peptide, 200 μ g of *L. waltii* peptide, or 100 μ g each of both peptides, in the presence or absence of Pronase were placed on a lawn of the indicated yeast. Although *S. cerevisiae* shows a halo of growth inhibition around all filter disks that is largely eliminated in the presence of Pronase, *L. waltii* does not show any detectable response

Figure 5.

Transformation of *L. waltii* with autonomous and integrative sequences. *L. waltii* cells were transformed with (A) pUC18:KanMX, (B) pUC18:KanMX : ARS, (C) pUC18:KanMX : CEN or (D) pUC18 : KanMX : CEN-ARS plasmid. Colonies from pUC18:KanMX : ARS transformed cells display a nibbled phenotype (Fig. B lower), whereas colonies from pUC18:KanMX : CEN-ARS transformed cells are smooth (Fig. D lower). (E) Schematic diagram of the fragment integrated into the *URA3* locus to create the *ura⁻* *L. waltii* strain. Images show growth of wild-type (WT) and *ura⁻* *L. waltii* streaked on the indicated media. (F) Schematic diagram of the fragment integrated at the *HIS3* locus to generate the *his⁻* *L. waltii* strain. Images show growth of wild-type (WT) and *his⁻* *L. waltii* streaked on the indicated media

Figure 6.

Mating and sporulation of genetically marked *L. waltii*. (A) Numerous tetrads were observed following coincubation of *MATa* and *MATα* cells on DYM medium for six days at 23 °C. Several presumptive shmoos were also observed under these conditions (lower right-hand panel). (B) Southern analysis of prototrophic colonies produced on YEPD (lanes a and b) and DYM (lanes c–f). Diploid colonies (a and b) show two alleles at the *HIS3* locus, as well as equal ratios of *MATa* : *MATα*. In contrast, lanes c–f are haploid segregants that show a single allele at the *HIS3* locus and a single idiomorph at the *MAT* locus. Ploidy was confirmed by flow cytometry (data not shown)

Figure 7.

CHEF gel analysis of the *L. waltii* genome. A longer and shorter exposure of the ethidium bromide (EtBr) stained CHEF gel of *L. waltii* chromosomes are shown in the left two lanes. Four individual bands plus what appears as a doublet at the bottom can be seen on the EtBr stained gel. The Southern blot was probed with unique probes for each of the eight chromosomes, as indicated by the number above each lane. Lane VIIa shows an unexpectedly large chromosome VII, indicating a large chromosomal change in this particular clone. Chromosome VIII appears as the large diffuse band at the top of the gel, and hybridizes strongly to the probe for the *L. waltii* rDNA repeat

Figure 8.

The *L. waltii* Genome Browser. (A) The browser includes three idiormorphs of chromosome V: the original assembly of chromosome V lacking the mating locus (middle panel), chromosome V *MATa* (top panel), and chromosome V *MATα* (bottom panel). The mating locus sequence for *MATa* or *MATα* was inserted into the gap in the original chromosome V assembly. (B) The *L. waltii* genome browser displays the location of *L. waltii* centromeres, sequence gaps, tRNAs, and ORFs. It can display homologs in *S. cerevisiae* annotated by Kellis *et al.* (2004) or Byrne and Wolfe (2005) as well as homologs in the yeast ancestor (Gordon *et al.*, 2009). The *S. cerevisiae* homologs can also be visualized as syntenic blocks. The Kellis and Wolfe *S. cerevisiae* homologs are almost identical but do differ in dynamic regions of the genome like the telomeres. In the region of chromosome IV shown, there is a discrepancy between the Kellis and Wolfe *S. cerevisiae* homologs at *L. waltii* gene 17 909

Table 1*L. waltii* growth rates at 23 °C or 30° in various media

Medium	Temperature (°C)	Mean doubling time (min)	SD (min)	Number of replicates
YC+2% glucose	23	124.8	31.3	5
YEP+2% glucose	23	134.7	14.6	7
YEP+3% glycerol	23	160.8	22.5	8
YC+2% glucose	30	74.1	6	4
YEP+2% glucose	30	83.2	2.3	3
YEP	30	112	6.1	3
YEP+3% glycerol	30	106.6	NA	1
YC+3% glycerol	30	1155.2	NA	1