January 2021

The Phylogenetic Analysis Of Genes Encoding Specific Steps In The Glutathione Pathway Within Industrial Beer Brewing Strains: Saccharomyces Cerevisiae, Saccharomyces Pastorinus, And The Spoilage Microbe Brettanomyces

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THE PHYLOGENETIC ANALYSIS OF GENES ENCODING SPECIFIC STEPS IN THE GLUTATHIONE PATHWAY WITHIN INDUSTRIAL BEER BREWING STRAINS: SACCHAROMYCES CEREVISIAE, SACCHAROMYCES PASTORINUS, AND THE SPOILAGE MICROBE BRETTANOMYCES

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Submitted to the Faculty of the Graduate School of
Eastern Kentucky University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

2021

DEDICATION

These works are dedicated to Georgia Mae Johnson, Karen Overstreet, and Lorenzo Ray Smith Jr.

ACKNOWLEDGEMENTS

The completion of this study would not have been possible without the expertise of my thesis committee which included: Dr. Patrick J. Calie, Dr. Christian M. Paumi, Dr. Jamie D. Fredericks, and Dr. David M. Hayes. I would also like to thank the Eastern Kentucky University faculty for their continued support, especially Dr. Marcia M. Pierce, Dr. Tanea Reed, Dr. Brett T. Spear, Dr. Lindsay Calderon, Dr. Patti Costello, Dr. Margaret W. Ndinguri, and Dr. Lisa Middleton.

ABSTRACT

The Glutathione pathway, (*GSH*) is an antioxidant system in yeast that increases cell viability and contributes to the production of desirable beer flavors during industrial fermentation. Despite its importance, studies using the *GSH* pathway: *GSH1*, *GSH2*, *GLR1*, and *SOD1* genes, to trace the evolutionary history of beer strains are lacking. As a result, the investigator sought to elucidate the phylogenetic relationships between four commonly used industrial beer strains: California Ale, London Ale, Oktoberfest, and *Brettanomyces bruxellensis* through single-gene sequencing analysis of the *GSH* pathway. It was hypothesized that the actions of these *GSH* genes are unique and potentially upregulated in beer brewing yeasts when compared to non-brewing yeasts strains. In order to assess this theory, *GSH* pathway genes from the experimental industrial strains, were sequenced in order to demonstrate that brewing yeast exhibit identical *GSH* pathway sequences as an adaptation to their shared brewing environments.

Following genome sequencing and phylogenetic analyses, the investigator found that these strains possessed identical *GSH* pathways as a result of various physiological adaptations and prolonged use within industrial settings. The investigator's results highlight the evolutionary significance and functionality of *GSH* pathway genes and demonstrate the essentialness of antioxidant activity in industrial yeast strains. These results also revealed that important strain related associations can be inferred through an analysis of essential metabolic pathways.

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Introduction

1.1. Beer brewing

In beer brewing, the selection of the appropriate starter culture is the key to a successful fermentation, and by selecting yeast strains with the desired fermentative and metabolic traits, brewers are able to improve the overall process and produce high quality beer products.

During fermentation, yeasts extract energy from sugars such as glucose, fructose, and sucrose, resulting in the production of carbon dioxide, (CO_2) and ethyl alcohol—giving beer both its alcohol content and its carbonation [7]. However, throughout this process, strains encounter a number of stressors associated with high sugar concentrations, aeration, ethanol toxicity, and elevated temperatures which contribute to the production of Reactive Oxygen Species (ROS) and ultimately a decrease in cell viability [28].

Reactive Oxygen Species Effect on Fermentation

ROS are highly reactive free radical intermediates that include singlet oxygen ($1O_2$), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2), and superoxide anion (O_2 −). When produced in excess, cellular antioxidant mechanisms become inundated, inducing a systemic imbalance known as oxidative stress [22]. In this state, ROS oxidize nitrogenous bases and phosphates, resulting in single and double stranded breaks in the DNA. The cell's inability to prevent these damages results in mutations and apoptosis that contribute to sluggish fermentations and cellular ageing [17].

A Brief Introduction to Industrial Fermentation

To begin fermentation, a starch source (commonly barley or grain) is steeped (soaked in water to extract fluid) and the resulting sweetened liquid known as wort, is fermented with yeast. Temperature preference varies based on the type of beer produced. In the case of ale beers, the wort is maintained at a temperature of approximately 20 °C for about two weeks while lagers are maintained at 8 °C for about six weeks [1]. Since fermentation produces a substantial amount of heat, the brewing tanks are cooled constantly in order to maintain the proper temperature [1].

- (These fluctuations in temperature have been found to increase oxidative stress and the overexpression of antioxidant enzyme genes in beer brewing yeasts [1]).

When wort is first added to the yeast, the specific gravity, (A specific gravity reading refers to the total amount of dissolved solids in water. In regards to beer, these dissolved solids are sugars) of the mixture is measured. Thereafter, the specific gravity is measured again to determine how much alcohol is in the beer, and to know when to stop fermentation [38]. The fermenter is then left open for aeration, (Oxygen is

generally required in order for yeast to synthesize sterols and the unsaturated fatty acids responsible for cell membrane biosynthesis; insufficient sterol and fatty acid synthesis results in inferior membranes and alcohol intolerance [16]) and then sealed off from the air except for a long narrow vent pipe, which allows carbon dioxide to escape from the fermenter. This constant flow of CO2 through the pipe prevents outside air from entering the fermenter, thus reducing the threat of contamination [38].

- (Although important, aeration has also been shown to increase oxygen solubility in fermentation media, thereby potentially enhancing ROS production [38]. Therefore, to achieve highly efficient ethanol production, fermentation with controlled aeration and antioxidant supplementation is often required [38]).

When fermentation is nearly complete, most of the yeast will settle to the bottom of the fermenter [1]. The fermenter's cone shaped bottom makes it easy to collect and remove yeast, which are then saved and repitched in subsequent batches of beer [6]. Yeast can be reused any number of times before replacement. Yeasts are replaced once noticeable differences in taste are detected within the final beer product [20]. Alterations in taste are indicative of novel mutations occurring within the yeast genome [31].

Upon completion, the beer is cooled to approximately 0° C. This helps the remaining yeast settle to the bottom of the fermenter, along with other undesirable proteins that filter out of the solution at lower temperatures [20].

1.2. The Glutathione Pathway

The ability of beer strains to effectively adapt to these fluctuations in pH, dissolved oxygen concentration, nutrient supply, ethanol concentration, temperature, etc., seems to be a unique characteristic of many of the industrial strains used today. Of the many ways that yeast have to cope with these various stressors, one of the most important is the glutathione (*GSH*) pathway [28]. The *GSH* pathway is comprised of four major endogenous antioxidant genes: glutamate-cysteine ligase (*GSH1*), glutathione synthetase (*GSH2*), glutathione reductase (*GLR1*), and superoxide dismutase (*SOD1*). The *GSH* pathway acts by neutralizing the harmful effects of Reactive Oxygen Species, (ROS) via the donation of reducing equivalents [28].

Biosynthesis and Introduction to the GSH Pathway Genes

GSH1, (Chromosome 10) & *GSH2*, (Chromosome 15)
Two ATP-dependent reactions synthesize *GSH* from amino acids.

1. L-glutamate + L-cysteine + ATP → γ-glutamyl-L cysteine + ADP + Pi

   Gamma-glutamylcysteine is synthesized from the amino acids L-glutamate and cysteine by the enzyme gamma-glutamylcysteinesynthetase [49].

2. γ - glutamyl-L-cysteine + L-glycine +ATP→ *GSH* + ADP +Pi

   Glycine is joined to the C terminus of gamma-glutamylcysteine via the enzyme *GSH2*.

   GSH1 is synthesized intracellularly through two consecutive ATP-dependent reactions catalyzed by γ-glutamylcysteinesynthetase. *GSH1* catalyzes the formation of γ-glutamylcysteine. *GSH2* then acts by ligating the proteinogenic amino acid, glycine, to the newly formed dipeptide, γ-glutamylcysteine to form *GSH*. *GSH* neutralizes free radicals by donating a reducing equivalent to produce its oxidized form, glutathione disulfide, (*GSSG*) [42].

   Once oxidative conditions are induced, high levels of *GSH* are necessary for cell viability and beer stability post bottling [28]. Preliminary studies concluded that below a critical threshold corresponding to about 10% of the normal value of the *GSH* cellular pool, viability of *Saccharomyces cerevisiae* cells was strongly impaired [38]. Several studies have also found that *GSH1* mutants unable to synthesize L-γ-Glutamate-L-cysteine ligase, (required for formation of γ-glutamylcysteine) are hypersensitive to hydrogen peroxide and super anions [28].

GLR1, (Chromosome 16)

Glutathione reductase (*GLR1*) is a reducing agent that mediates the conversion of *GSSG* to its reduced form, *GSH*, thereby maintaining the intracellular redox environment represented by the quantification of *GSH*: *GSSG* ratio, (Cellular *GSH* homeostasis: 98% *GSH* and less than 2% *GSSG*). *GLR1* is not essential for normal aerobic growth but is required for viability when exposed to oxidative stress conditions [26]. Studies have found that the overexpression of *GLR1* resulted in increased oxidative stress tolerance while *GLR1* mutants demonstrated oxidant sensitivity [38].

SOD1 (Chromosome 10)

Superoxide dismutase (*SOD1*) interacts with copper chaperone (*Ccs1*) binding domains to facilitate the conversion of superoxide anions to less toxic hydrogen peroxide and molecular oxygen [61]. *SOD1* mutants have demonstrated an inability to induce antioxidant activity, resulting in sensitivity to heavy metals, defective sporulation, and senescence. *SOD1* deletions have also been found to dramatically reduce the replicative life span of yeast [39]. *GSH* acts as a redox buffer, protecting yeast *SOD1* against oxidation. Thus, increases in cellular longevity are *SOD1*/ *GSH* dependent [61].

1.3. Statement of Importance

GSH pathway function is a desirable characteristic for yeast employed in industrial settings, and high-stress brewing environments may have exerted a natural selection pressure to evolve these yeasts into highly efficient *GSH*- producing strains. Due to the *GSH* pathway's importance, the investigator hypothesizes that the actions of the *GSH1*, *GSH2*, *GLR1*, and *SOD1* genes are unique and even potentially upregulated in brewing yeasts when compared to non-brewing yeasts strains. In order to assess this theory, *GSH* pathway genes from various industrial beer strains, particularly: London Ale, (*Saccharomyces cerevisiae*), California Ale (*Saccharomyces cerevisiae*), Oktoberfest (*Saccharomyces pastorianus*, Weihenstephan 34/70), and *Brettanomyces bruxellensis* will be sequenced in order to demonstrate that brewing yeasts exhibit identical *GSH* pathway sequences as an adaptation to their shared brewing environments [49].

Several studies have analyzed yeast populations via genome sequencing. However, these studies prioritize wine strains, with no lab examining GSH pathway genes' role in influencing evolutionary relationships amongst industrial beer strains [20]. The technique used in these phylogenetic investigations has been that of Whole Genome Sequencing, (WGS); a method which determines the complete DNA sequence of an organism's genome [46]. Although WGS methods are comprehensive and effective, it has been found that it is rarely necessary to use complete sequence databases to identify close homologs as more sensitive searches can be limited to single gene sets from evolutionary close organisms [48]. Because of its sensitivity and the slower rate of change in these conserved sequences, (*i.e.GSH* pathway), single-gene sequencing was employed to detect homologous sequences [48].

1.4. Industrial Strain Divergence

Beer brewing yeasts have been found to exhibit distinctive physiological properties that are generally not found in non-beer brewing yeasts. Previous studies have demonstrated that ale and lager strains possess higher antioxidant defenses and display higher fermentative capacity when compared to non-brewing strains [7]. In general, non-brewing strains display a low GSH/GSSG ratio which is considered a negative factor for a healthy redox state. These studies suggest that deficient oxidative defenses in non-brewing yeasts may be a limiting factor for adaptation during industrial fermentation [7].

1.5. Biography of *Brettanomyces bruxellensis*

Brettanomyces bruxellensis is a genus of feral yeast traditionally used in the production of Lambic and Flanders ales [12]. Although originally classified as a spoilage microbe, it has risen to prominence due to its contribution to the peculiar flavors present

within specialty beers [12]. Comparative analyses of *Brettanomyces bruxellensis* unveiled several genomic properties that confer important advantages over other yeast species such as oxygen consumption, dextrin metabolism, and elevated stress tolerance [9].

Additional advantages include a lineage specific duplication of oxidoreductase genes [51]. This duplication event may have reflected a strategy evolved to enable *Brettanomyces bruxellensis* survival in anaerobic brewing conditions and also might explain its capacity to produce key aromatic compounds such as isovaleric acid, (produces a pungent cheesy flavor in beers; this flavor is often indicative of contamination, if not intentional) [51].

Directed Evolution and its Influence on Beer Brewing Strains

While the physiological behavior of non-beer brewing yeasts is exclusively dedicated to survival and reproduction, most industrial fermentations require the maximization of strain characteristics that may not be beneficial in natural environments. Several techniques have therefore been developed to artificially enhance strains that perform better in industrial settings, with the directed evolution method being the most notable [13].

Directed evolution is a method used in genomic engineering that mimics the process of natural selection to steer proteins or nucleic acids toward a user-defined goal [13]. In general, a population of cells is grown under continuous selection for the genotype/ phenotype of interest for many generations (cell divisions).

In fact, it has been found that in applications where yeasts are continuously used for longer time periods, for example *Saccharomyces* strains in beer fermentations; these populations have been subjected to unintentional directed evolution, yielding strains with positively adjusted genotypes that are suitable for thriving in industrial environments [13].

MATERIALS AND METHODS

2.1. Materials

This protocol provides a detailed description of how to perform qualitative isolation methods, streak plate method, DNA isolation, PCR product preparation, gel electrophoresis, DNA gel extraction, Sanger sequencing, multiple sequence alignments, and phylogenetic tree construction. This method employs specific reagents purchased exclusively from White Labs, Pure Yeast & Fermentation (Asheville, North Carolina), Zymoresearch (Irvin, California) and Eton Bioscience (Durham North, Carolina).

The supplies used during this experiment included: test tubes (25), petri dishes (50), distilled water, glass coverslips (10), microscope, incubator, refrigerator, Bunsen burner, Yeast Extract Peptone Dextrose, (YPD), YPD solid medium, inoculating loop, tweezers, scapula, micropippete, non-filtered 200 μl pipette tips, SeaKem ® LE Agarose, QIAGEN CLC Sequence Viewer 8 Software, ClustalX Multiple Alignment of Nucleic Acid and Protein Sequences Software, DNA Base Assembler Software, (Contig Generator), YeaStar Genomic DNA Kit, Zymoclean ™ Gel DNA Recovery Kit, and ZymoTaq Premix. Four different yeast genera obtained and pre-labeled from Whitelabs were used throughout this investigation: London Ale/ California Ale, (*Saccharomyces cerevisiae), Brettanomyces bruxellensis*, and Oktoberfest. The identification of these strains were certified by Whitelabs through genotyping and additional on-site tests which ensured that strains were free from aerobic/ anaerobic bacteria and wild yeast contamination.

Control(s): Strawberry Ale, (ITS Primers) were used to ensure that the gel electrophoresis procedure was performed properly and that bands representing molecules of different sizes could be accurately detected. YPD medium was also used to ensure that growth parameters remained consistent among each strain sample.

Oktoberfest, (WLP820), London Ale, (WLP013), California Ale, (WLP001), and *Brettanomyces bruxellensis*, (WLP650), strains were provided by Eastern Kentucky University's Chemistry Department and ordered from White Labs Pure Yeast & Fermentation Corporation and were chosen due to their varied metabolic mechanisms, growth rates, and industrial applications.

Yeast Strain Validation

Upon receiving yeast samples from White Labs Pure Yeast & Fermentation, serial dilution was performed in order to reduce the concentration of the microscopic cells within the samples. Next, the cells were inoculated into a liquid YPD broth culture. Once grown (24 hours-36 hours), cells were isolated on YPD agar via the streak plate method, (used for the isolation of pure colonies). Yeasts were differentiated based on known morphological characteristics associated with each strain, (e.g. shape, opacity, size).

Strain identification was also confirmed through the use of Internal transcriber spacer, (*ITS*), *ITS1* and *ITS4* primers. *ITS* sequence comparison is widely used in molecular phylogeny and taxonomy due to the fact that it generally shows more variation than ribosomal sequences, making them popular for the identification of species and strains [3]. This can be explained by the relatively low evolutionary pressure acting on such non-coding spacer sequences [3].

During the experiment all strains went through "repeat runs", (sequenced multiple times to ensure validity). All repeats were not included in this experiment and strains used were specifically selected based on their accordance with ETON's quality scores, (with high quality sequences receiving a high-quality control score, highlighted by a green marker).

Streak-Plate Method

Individual yeast strains were aseptically streaked onto separate YPD labeled agar plates using the following Streak-Plate Method:

An inoculating loop was sterilized after being placed in the Bacti-Incenerator IV, (Bunsen burner) and gently touched to the agar to cool. Next, the inoculating loop was used to obtain a single colony from the sample plate. The inoculating loop was gently drawn several times along the surface of the agar in a zig-zag pattern that covered one-third of the plate. The inoculating loop was re-sterilized by being placed into the Bunsen burner and cooled by touching it to a sterile portion of the agar. The inoculating loop was drawn across the previous streak marks into another zig-zag pattern on a new portion of the plate. The inoculating loop was not sterilized again and was drawn across the newest streaked marks in a repeated pattern across the final sterile portion of the plate. The lid was placed back on the cover, with the agar facing down to avoid droplet contamination. The plate was then labeled appropriately and set aside. This process was repeated with each yeast sample.

2.2. Liquid Yeast Culture Production

The agar plates were placed into an incubator at 30 °C for 24-72 hours until individual colonies were observed. Twenty-five milliliters, (mL) of YPD was obtained and pipetted into a 50 mL FisherBrand conical vial. Using a sterile inoculating loop, a single colony was obtained from the developed streak plate and added to the conical vial. The conical vial was then placed in a New Brunswick Scientific Incubator Shaker for 24-48 hours to allow liquid cultures to grow. Once complete, the liquid yeast cultures were used to isolate genomic DNA by following the YeaStar Genomic DNA Kit, Protocol I [Protocols cited from YeaStar Direction Manual]:

Protocol I

The kit works with either fresh cells or aged cells in either plates or liquid cultures. The following procedure is based on 1-1.5 ml culture (1-5 x 10^7 cells). Increasing the amount of cells above the recommended level may cause overloading of the system.
1. Spin 1-1.5 ml of cells down briefly or centrifuge at 500 g for 2 minutes. Remove the supernatant completely.
2. Add 120 microliters (μl) of YD Digestion Buffer and 5 μl of R-Zymolyase™ (RNaseA+Zymolyase™). Resuspend the pellet by vortexing and incubate at 37°C for 40-60 minutes.
3. Add 120 μl of YD Lysis Buffer*. Mix well by gently vortexing. You can vortex hard for 10-20 seconds after adding YD Lysis Buffer. This will increase your DNA recovery but may result in shorter genomic DNA ranging from 20-35 kb. However, most of the DNA will remain more than 35 kb.
4. Add 250 μl of chloroform. Mix thoroughly for 1 minute.
5. Centrifuge in a tabletop centrifuge at >10,000 rpm for 2 minutes.
6. Load the supernatant onto the Zymo-spin III column and centrifuge at >10,000 rpm for 1 minute.
7. Add 300 μl of DNA Wash Buffer and centrifuge for 1 minute at ≥10,000 rpm to wash. Add another 300 μl of DNA Wash Buffer to repeat the wash and centrifuge for 1 minute. Transfer the Zymo-spin III column to a new 1.5 ml centrifuge tube and add 60 μl of water or TE directly onto the membrane. Wait for one minute then centrifuge for 10 seconds to elute the DNA.
Contains beta-mercaptoethanol. * Contains Chaotropic salt. Irritant. Handle with care.
Note: Before starting, add 24 ml of 95-100% ethanol to the DNA
Wash Buffer.
8. Add 1 μl of eluted DNA to the Thermo Scientific NANODROP 2000 Spectrophotometer to ensure that DNA samples are free off contamination. A 260/ 280 ratio of ~1.8 is generally accepted as pure for DNA; expected 260/ 230 values are commonly in the range of 2.0-2.2.
9. Repeat procedure with each subsequent DNA strain, (*e.g., Saccharomyces cerevisiae,* Oktoberfest, *Brettanomyces bruxellensis*)
After obtaining 260/ 280 ratios of at least 1.8 *n*g/ μl and a 260/ 230 ratio, a PCR reaction was setup using the following parameters:

PCR Protocol: Primers ordered from Integrated DNA Technologies (IDTDNA) (Skokie, Illonois) for London Ale, California Ale, and Oktoberfest *GSH* pathway genes were designed using Strawberry Ale (Ascension Number: NC_001136.10) genomic sequences as a template.
Primer Activation, (Dilution): A 1/10 dilution was performed by diluting the 100 μM IDTDNA stock solution. 90 μL of distilled (DI) water and 10 μM of IDTDNA Primer Stock was added to a sterilized 1.5 mL centrifuge tube. Ready-made PCR tubes were assembled by inserting Zymotaq Premix, Forward Primer, Reverse Primer, DNA template, and ddH_2O, (DNase/ RNase Free).

2.3. Gel Electrophoresis

Upon removal from the PCR machine, gel electrophoresis was performed to detect and separate DNA fragments based on their size. Agarose gel was prepared by placing 1 gram of SeaKem ® LE Agarose into a 250 mL flask. Next, 1 X TAE buffer was created from a 50 X TAE Buffer by diluting 20 mL of 50 X TAE stock solution into a 1000 mL beaker filled with 980 mL of Deionized water, (DIh20). The agarose/ DIh20 mixture was then placed in a microwave for 1 minute and 30 seconds. After allowing the mixture to cool for one minute, 2.5 μl of Ethidium Bromide, a fluorescent used to visualize DNA fragments, was placed into the flask. The agarose mixture was slowly poured into the gel tray and a horizontal gel comb, (used for sample separation and well formation) was placed in the warm mixture. The investigator then waited until the gel completely solidified, (10-12 min). The solidified gel was inserted into the apparatus with the precast wells in the gel faced towards the negatively charged anode, (The ortho-phosphate backbone of the DNA molecule is negative, therefore when placed in the electrical field, DNA fragments will migrate towards the positively charged anode). A sufficient amount of 1 X TAE Buffer was added to cover the surface of the gel, (980-1000 mL of 1 X TAE Buffer). Six μl of Invitogen ™ 1 Kb Plus DNA Ladder, (Unit size/ Concentration: 250 μg) was always inserted into the first well lane to identify the approximate size of the DNA fragments. Ten μl of DNA sample and two μl of Invitrogen ™ BlueJuice Gel Loading Buffer, designed for easy loading and tracking of DNA samples in agarose gels were mixed and inserted into the subsequent lanes. DNA samples were run at 175 millivolts, (mV) for 45 minutes. The migrated DNA samples were then analyzed under 302 nanometer(*nm*) midrange UV light, to ensure that the observed bands corresponded with the known base pair length, (bp) of the gene in question.

2.4. Zymoclean Gel DNA Recovery Kit

GSH pathway PCR amplicons were recovered using the Zymoclean Gel DNA Recovery Kit. The protocol proceeded as follows [Protocols cited from Zymoclean Directional Manual]:

Buffer Preparation

Before starting: Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml DNA Wash Buffer concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml DNA Wash Buffer concentrate.
DNA Wash Buffer included with Gel Recovery Kit (D4001S) and Sample Gel Recovery Kit (D4001T) is supplied ready-to-use and does not require the addition of ethanol prior to use.
Protocol
All centrifugation steps should be performed between 10,000 - 16,000 x g.
1. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of Agarose Dissolving Buffer (ADB) to each volume of agarose excised from the gel (*e.g.*, for 100 μl (mg) of agarose gel slice add 300 μl of ADB.
3. Incubate at 37-55 C for 5-10 minutes until the gel slice is completely dissolved.
For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 μl agarose, 300 μl ADB, and 100 μl water).
4. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 μl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 μl DNA Elution Buffer or water directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

Ultra-purified DNA is now ready for use. Purified DNA samples were sent to ETON Bioscience Inc. (Durham, North Carolina) for Sanger sequencing. Reverse transcripts for the complementary strand were generated via The Sequence Manipulation Bioinformatics Site and contigs were assembled using DNA Base Assembler Software, which is specifically designed for DNA sequence conversion (converts a DNA sequence to its reverse-complement counterpart), contig editing, and mutation detection [15].

2.5. ClustalX 2.0

To examine relatedness among industrial yeasts strains, the *GSH* pathway genes of *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Brettanomyces bruxellensis* were sequenced. This collection included two ale strains, London and California Ale, one lager strain, Oktoberfest, and one feral yeast strain, *Brettanomyces*

bruxellensis. ClustalX, a multiple sequence alignment program was employed to align sequences using a technique that progressively builds multiple sequence alignments from a series of pairwise alignments. To identify core conserved elements in the *GSH* pathway; glutamate-cysteine ligase (*GSH1*), glutathione synthetase (*GSH2*), superoxide dismutase (*SOD1*), and glutathione reductase (*GLR1*) sequences from each strain were aligned to highlight identical regions common to each strain. Sequences were input in FASTA format using the FILE menu with all non-alphabetic characters being ignored except "-"which was used to indicate a GAP in the sequence of one genome relative to its counterpart in the pairwise view. GAPS are classified as indels, a molecular biology term for insertions or deletions in the genome of an organism [35].

The MULTIPLE ALIGNMENT MODE was then selected in order to visualize conserved sequences. The ClustalX format included a plain-text key to annotate conserved columns of the alignment, denoting conserved sequences (*), conservative mutations, (:), semi-conservative mutations, (.) and non-conservative mutations (), [35].

The ClustalX software indicated quality alignments by plotting a 'conservation score' for each column of the alignment [35]. High scores indicated a highly conserved alignment, (A highly conserved sequence is one that has remained relatively unchanged far back up the phylogenetic tree and is extremely similar among the experimental organisms); while a low score was representative of low conservation, (indicates that gene lacks support in regards to being conserved. Low conservation may indicate a lack of functional value). Quality scores provided for standard visualization are presented below the alignment. For additional quality assurance, low scoring residues were highlighted to indicate sequence segments which scored poorly in the alignment. Low scoring residues were expected to occur at a moderate frequency (35 % or less [1]) in all sequences because of the steady divergence between strains as a result of evolution [5]. However, an excess or clustering of highlighted residues would be indicative of sequence misalignments. Partial or complete misalignments indicate that a given set of alignments is partly or completely unrelated to the other sequences [35].

2.6. CodonCode Aligner

CodonCode Aligner is a commercial application used for the assembly of DNA, sequence alignment, and editing on Mac OS X and Windows operating systems [56]. The CodonCode Aligner "End clipping" option acted by automatically removing low-quality sequences from the beginning and end of sequences by administering sequence quality scores to identify low-quality regions, (for sequence traces that do not have quality scores, CodonCode Aligner allows investigators to use the base calling program Phred to assess and assign sequence qualities) [56]. CodonCode Aligner also allowed the investigator to control the stringency of the trimming process through a variety of end clipping parameters which resulted in high quality sequences of similar and equal lengths.

The CodonCode Aligner "Assembly" option enabled the investigator to assemble the sequence fragments into a larger sequence by identifying overlaps between sample sequences. Samples that could be joined together were placed into contigs. Fragment joining had the potential to fail if the overlap contained too many discrepancies or if the samples did not share overlaps with other samples or contigs [56].

The "Compare Contigs" option found within the "Advanced Assembly" submenu was selected as it is designed for assessing phylogenetic studies [56].

CodonCode Aligner also provided several options for viewing amino acid translations. Protein translation for every sequence used in this investigation as well as the consensus sequences corresponded with the twenty standard amino acids. CodonCode Aligner was also able to show a protein translation of the investigator's consensus sequences for annotated coding regions. Typically, this display was used together with reference sequences imported from Genbank or EMBL-format [59].

GSH pathway amino acid sequences/ nucleotide sequences were aligned against a *Saccharomyces* reference sequence for each specific protein, with BLAST finding the corresponding sequence to the query in the NCBI database. In order to tell whether the alignment was sufficient and whether it portrayed a significant biological relationship, BLAST bit score and Expect values (E-value) were also analyzed. The bit score provided an indication of the quality of the alignment with higher scores being indicative of quality alignments. In general terms, this score is calculated from a formula that takes into account the alignment of similar or identical residues, as well as any gaps introduced to align the sequences [65]. The E-value gives an indication of the statistical significance of a given pairwise alignment with lower E-values being indicative of significant hits. For example, a sequence alignment that has an E-value of 0.05 means that this similarity has a 5 in 100 chance of occurring by chance alone [65]. In this particular investigation London Ale, California Ale, and Oktoberfest GSH pathway sequence alignments resulted in an E-value of 1^{-47} which is indicative of biologically meaningful results. This value is considered as a quality hit for homology matches [65].

Mega X Software and Configuring Genetic Distance Matrices

Genetic distance matrices were developed using the Mega X software. Mega X required multiple sequence alignment inputs in order to accurately measure the genetic distance between the sequences being classified, with distance often being defined as the fraction of mismatches at each aligned position [33]. From this analysis, phylogenetic trees were constructed.

3.1. Results and Discussion

Multiple sequence alignments were used to identify functionally important sites, (*i.e.*, *GSH* pathway) by locating conserved domains [35]. The CLUSTAL multiple sequence alignment format included a plain-text key to annotate conserved columns of the alignment, denoting conserved sequences (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations () [35].

High conservation scores represented by peaks and asterisks (*) were depicted continuously throughout each alignment indicating identical residues at their respective positions, (Fig.1). Shortened branch lengths depicted below the phylogenetic trees further reinforced these observations, as shorter branch lengths indicated little genetic change or fewer mutations [44]. Being that *GSH* pathway genes have remained relatively unchanged, despite natural genetic occurrences, (e.g., chromosomal rearrangements or genetic mutations) it is likely that these genes were conserved in each strain as an essential adaptation to their common industrial conditions [40].

Brettanomyces bruxellensis and the Presentation of Base Substitutions

In this study, one of the most significant indicators of dissimilarity was represented by a high frequency of substitutions. In general, base substitutions constitute less than 10% of all yeast mutations [68]. However, the proportion is significantly high when observing *Brettanomyces bruxellensis* GSH2 and GLR1 gene sequences as compared to *Saccharomyces* strains. The ratio of conserved nucleotides to substitutions within GSH2 and GLR1 alignments indicates that an important role is played by these alterations potentially in the loss or gain of function [34]. When observing the low scoring residues within *Brettanomyces bruxellensis* sequences, it appears that this strain is evolving characteristics suitable for surviving in oxygen limited brewing conditions.

Although somewhat domesticated, and clearly more efficient at fermenting beer in the latter stages of the fermentation process, *Brettanomyces bruxellensis* still displays traits associated with its natural niche [53]. *Brettanomyces bruxellensis* is naturally an aerobic species with its GSH pathway acting to synthesize glutathione and reduce GSSG solely under oxygen-present conditions [53]. However, since its recent employment within industrial settings, base substitutions associated with anaerobic activity likely occurred as an adaptation for growth and survival.

Another explanation for this finding could be attributed to the Wobble Hypothesis which explains how multiple codons are able to code for a single amino acid due to the less-precise base pairs that arise between the 3rd base of the codon and the first base of the anticodon [68]. When observing the *Brettanomyces bruxellensis* GSH2 and GLR1 sequences a degree of steric freedom or wobble is often noticed in the third position, while the first two nucleotides are spatially confined in order to provide for the decoding

of the mRNA codon into an amino acid [68]. This hypothesis may explain why the degenerate codons observed in the *Brettanomyces* sequences code for the same amino acids seen in the *Saccharomyces* strains.

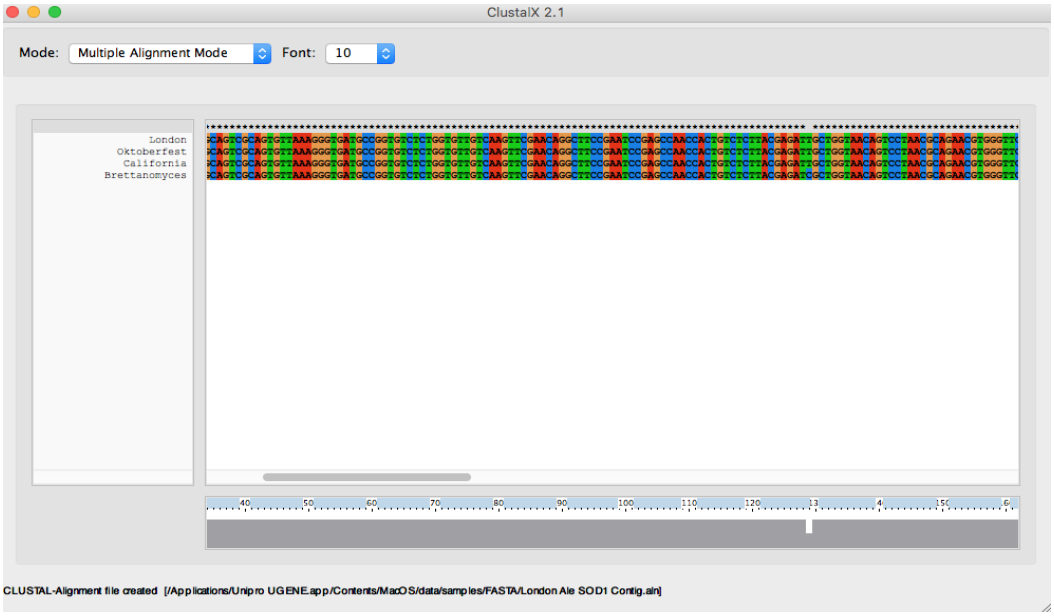


Figure 1: GSH Pathway: Highly Conserved Sequences

Partial multiple sequence alignment of the SOD1 gene using ClustalX 2.1 software. The SOD1 gene sequence was highly conserved as indicated by the asterisk symbols presented above the alignment. This partial alignment contains rich information concerning the structure/order of the aligned sequences and provides insight in regards to the evolutionary history of these strains.

Explanation for the Use of Out-Groups

In phylogenetics the out-group is used as a point of comparison and serves as a reference when determining evolutionary relationships [5]. To establish the optimal level of relatedness in regard to resolving evolutionary relationships of a clade, an outgroup should be chosen on the basis that it is not part of the ingroup but closely related enough so that meaningful relationships can be determined [5]. Therefore, four outgroups were selected, with each meeting the criteria required for each phylogenetic tree. The use of multiple outgroups has been found to be an effective means of testing the ingroup's monophyly, by acting as a buffer against the potential selection of poor outgroups [30].

Heterobasidan irregulare is a pathogenic root fungus used in the construction of the *GSH1* phylogenetic tree. This fungus is of considerable importance to the timber industry as it has caused considerable economic damage in several plantations across the United States [25].

Babesia ovata is a protozoan parasitic species which coexists alongside eukaryotic organisms such as cattle and ewes and was selected for the construction of the *GSH2* phylogenetic tree [55]. Infected animals may transmit the parasite to their offspring or to commercial products through the ingestion of contaminated milk [55].

Synchytrium microbalum are a basally derived phylum of fungi whose members are ubiquitous in aquatic and terrestrial habitats [32]. This species was used as the outgroup for the *GLR1* phylogenetic tree. *Synchytrium microbalum* grows in association with various vegetables and is a known contaminant of potato based alcoholic fermentation, (*i.e.*, potato/black wart disease) [32].

Debaroyomycea hansenii was used as an outgroup for the *SOD1* phylogenetic tree. This yeast species is commonly found in various types of cheeses and sausages and is known to destroy competitive yeast species through the release of toxins and mycocins [10].

Phylogenetic Trees

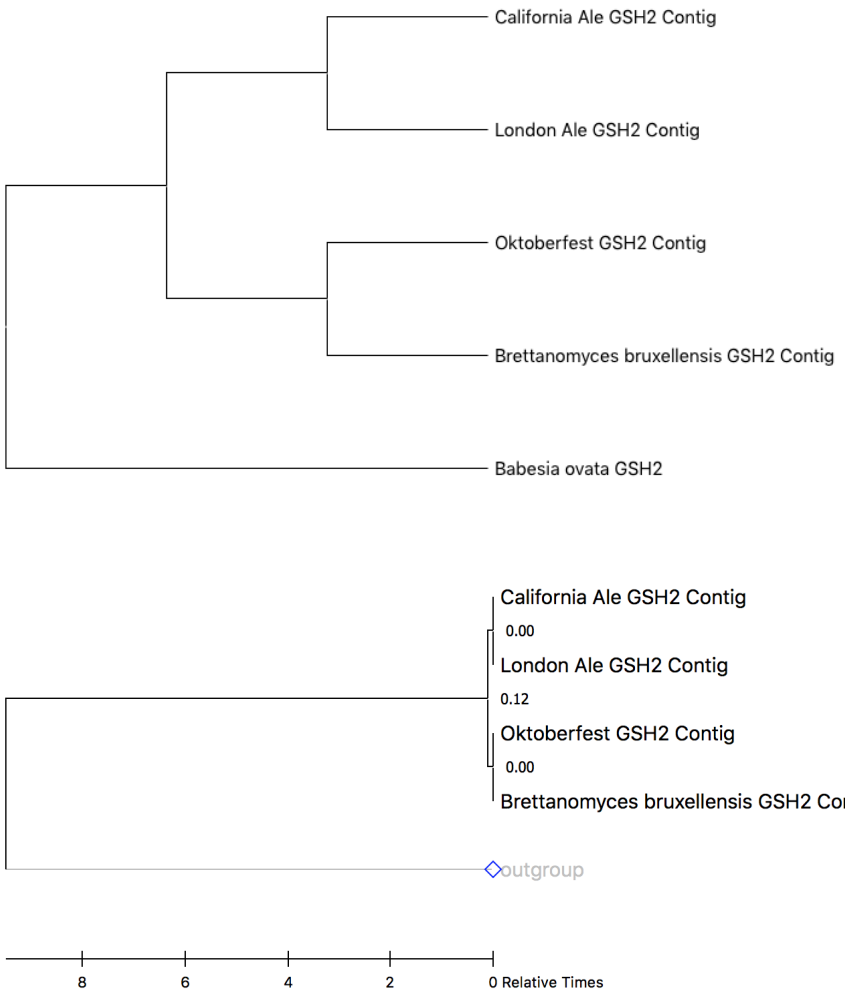
Phylogenetic analyses of all trees provided similar results, with London Ale, California Ale, and Oktoberfest *GSH* pathways being more closely related than to that of *Brettanomyces bruxellensis*. Geographic location and industrial application seem to account for these similarities and differences, as several studies conclude that yeast inhabiting similar environments possess distinct similarities associated with their restricted uses and geographic location [20]. Historical evidence also found that European immigrants, (including the Pilgrims) brought and brewed beer in the first American colonies between 1620 -1800, thus highlighting London Ale, California Ale, and Oktoberfest strains specific geographic region and prolonged subjection to similar selection pressures within both European and American brewing niches [63].

As expected, California and London Ale *GSH* pathways were found to be more closely related than to that of the Oktoberfest (lager) *GSH* pathway. These results are reflective of the different brewing parameters (temperature and flocculation) that the two strains are subjected to; as flocculation behavior and fermentation temperatures have been found to influence *GSH* pathway activity by either reducing or increasing it depending on the intensity of the stress response induced [24].

Yeast Evolution

Evolutionary change involves the substitution or alteration of new versions of genes for old ones, with new genes arising by mutation and coming to predominate because of favorable selection [13]. Thus, a series of evolutionary changes involves a progressive accumulation of genetic change in the DNA [13]. Strains that are more closely related therefore accumulate a smaller number of evolutionary differences [13]. This is indeed what is seen in our observations, as strains used in similar applications possess less variation or mutations within their *GSH* pathways. This regular pattern of change shows that yeast strains not used for beer brewing purposes or generally under

different conditions have noticeably different *GSH* pathways because the environment/ applications (in this case industrial breweries/ human selection) dictate the direction and extent of change between sequences [31].



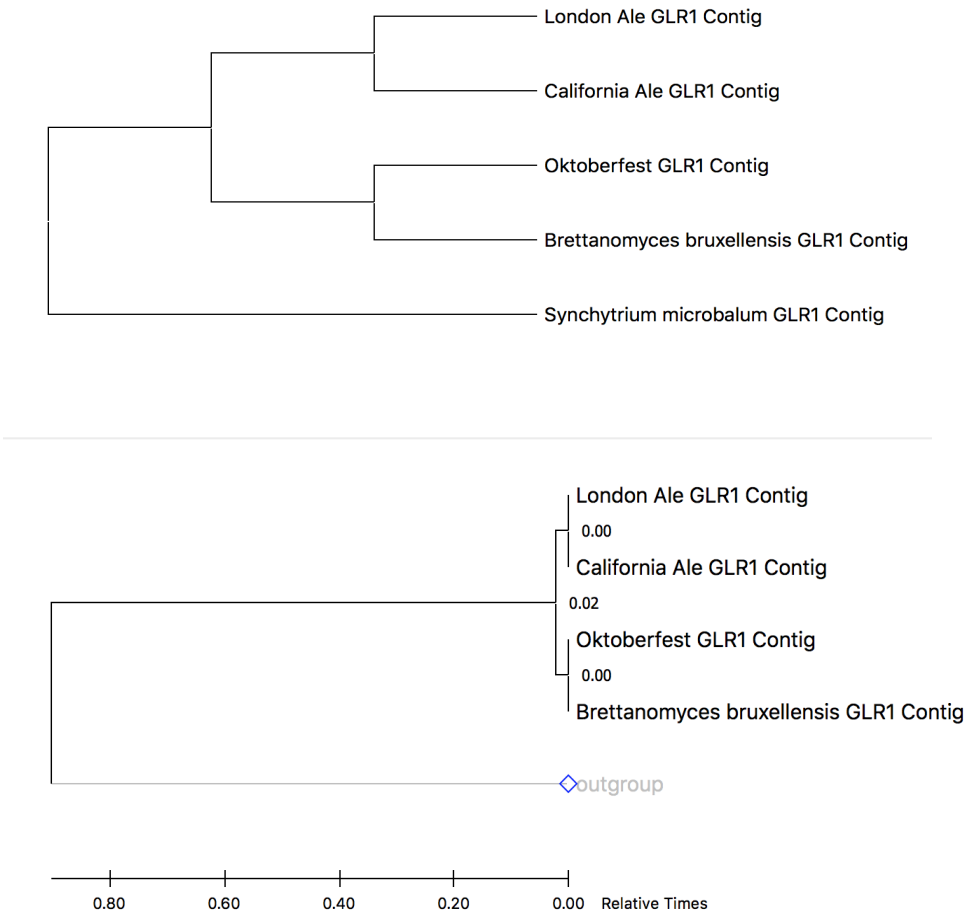


Figure 2. Discerning Outgroup and Ingroup Relationships

As expected, beer brewing strains were found to be more closely related than non-beer brewing strains, which was represented here by the outgroups, *Babesia ovata* (*GSH2*) and *Synchytrium microbalum* (*GLR1*). All branch lengths were also relatively short, indicating minor to negligible genetic change within these conserved genes.

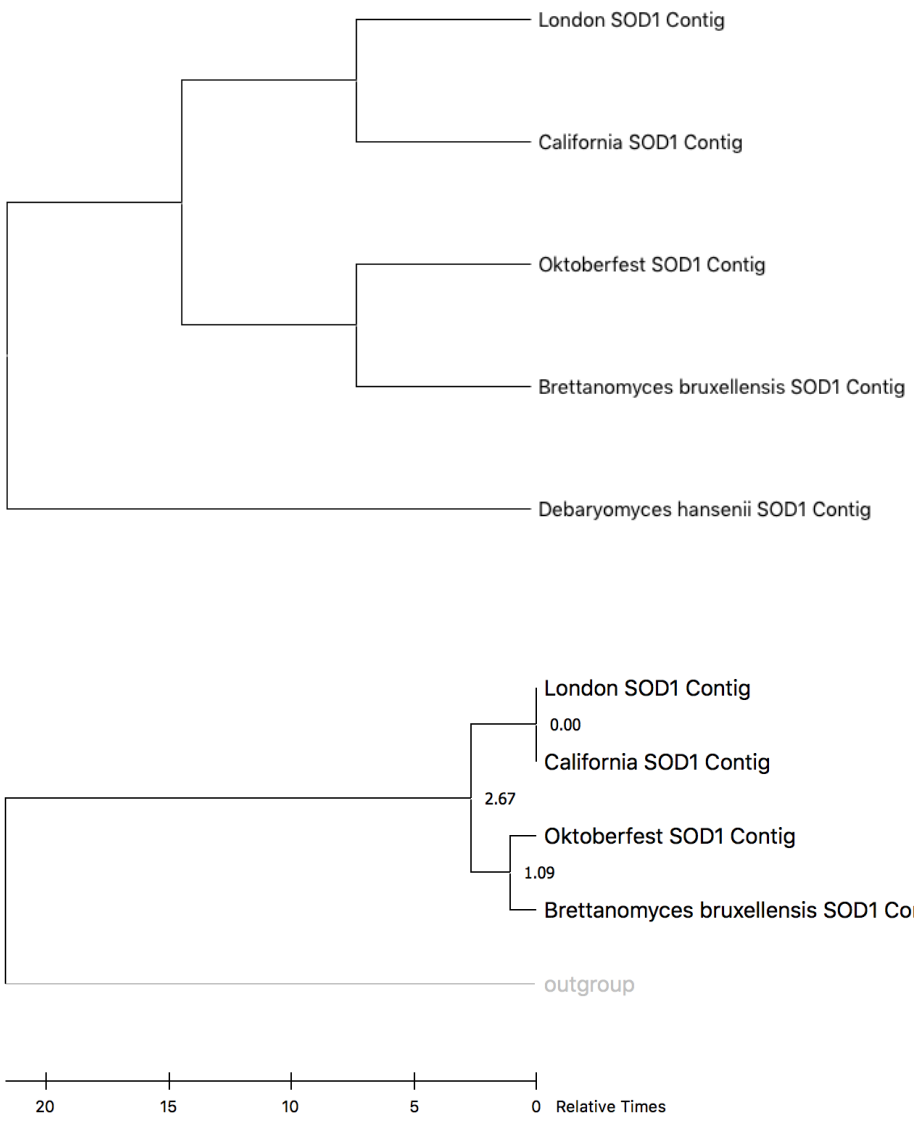


Figure 3: Moderate Genetic Change & Branch Lengths

According to branch lengths, each strain did not undergo many genetic changes. Branch lengths represented by scale bars indicate genetic change, with longer branches indicating that more genetic change or divergence has occurred. The measure of genetic change was calculated by estimating the average number of nucleotide substitutions per site.

Differentiation in GSH1 Phylogenetic Tree

The *GSH1* phylogenetic tree (Fig. 4) included three strains; London Ale, California Ale, and Oktoberfest rather than the four strains used consistently throughout the experiment. *Brettanomyces bruxellensis GSH1* was not observed on any of the sample gels resulting in the investigator's decision to exclude it from this experiment. This may be attributed to a failure of the primers to anneal to the *Brettanomyces* template; however, more research will be needed to support this conclusion [36].

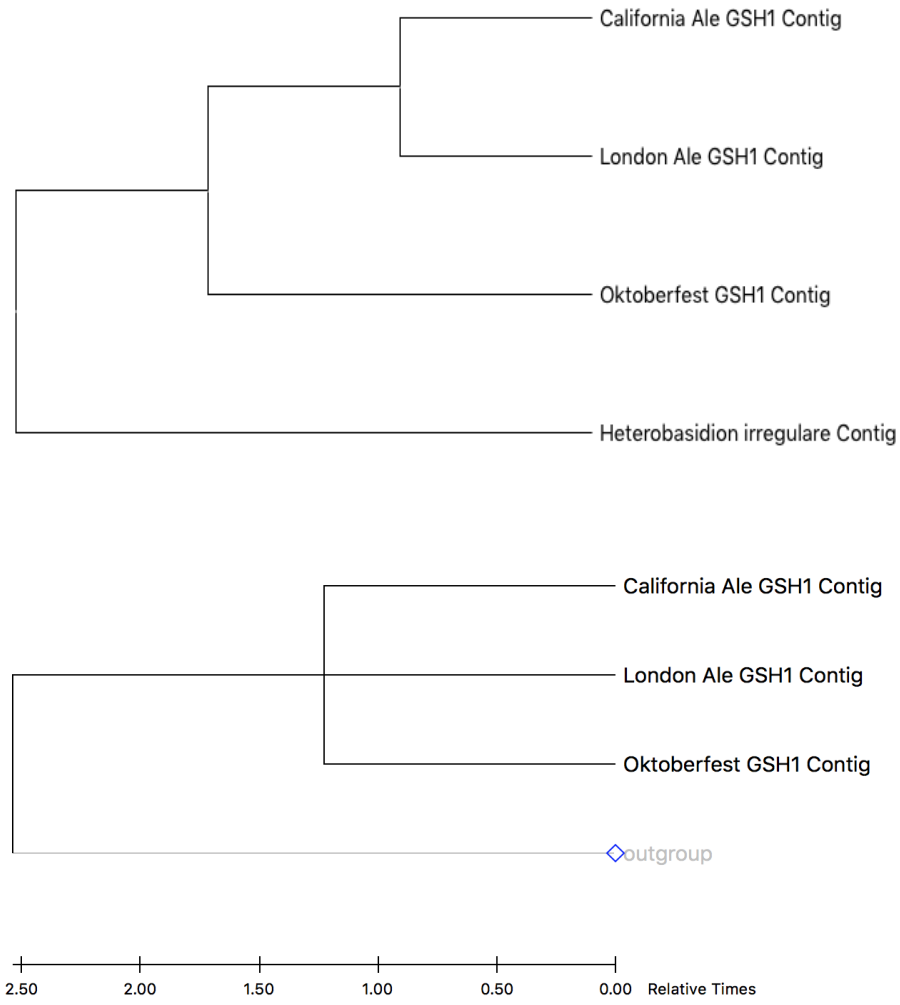


Figure 4. GSH Pathway as the Focal Point

The *GSH1* phylogenetic tree was constructed using the pathogenic fungus *Heterobasidion irregulare*. Ale and lager strains were once again observed clustering together, thus further reinforcing the aforementioned hypothesis.

Interpreting Branch Lengths

One of the major goals of phylogenetic systematics is to accurately estimate divergence among species and clades and although adequate models are necessary for accurate branch length estimation, they provide no guarantee of accurate estimates [44]. Substitution models specify only relative rates, but the absolute, average rate of substitutions per site along each branch must still be estimated from the data. Therefore, specific features of the dataset such as the number of taxa or the sequence length all likely impact branch length estimation [44]. However, the effects of these variables, on branch length estimation have not been fully explored and may even potentially account for the differences in branch lengths observed in the *GSH* pathway.

Furthermore, though our hypothesis is supported by literature in results and theory, it has been found that genes operating within the same pathway can vary in selection pressures [30]. Hence it should be noted that correlation in evolutionary rates, (*e.g.*, branch lengths) does not necessarily occur equally amongst all genes within the pathways [23].

[Previous studies have suggested that branch lengths can be estimated most reliably within a Machine Learning (ML) framework [33]. By employing the MEGAX software, the investigative team sought to employ the most available and reliable ML statistical framework in which to estimate branch lengths] [33].

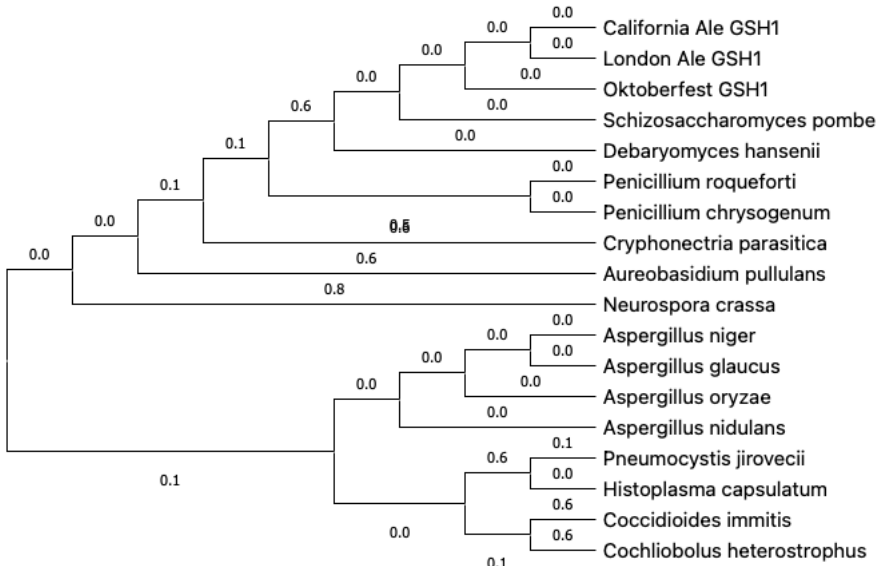
An Extended Analysis of Beer Brewing Strains and Non-Beer Brewing Strains

To further examine the evolutionary history of industrial yeasts, we included an additional representative set of 17 previously sequenced Ascomycota yeast strains from various sources. This collection included 4 strains isolated from spontaneous fermentations, 2 wine/fruit juice strains, 7 bioethanol strains, and 4 laboratory strains. After de novo assembly of each of the genomes, we inferred a maximum-likelihood phylogenetic tree based on codon alignments. Trees constructed from the original and extended datasets were congruent and further confirmed that the evolutionary divergence of industrial yeast are shaped by both geographic location and industrial application. [See

Table 4. for a list of strains sequenced and consensus PCR conditions-[Extended Genome Analysis: Strains]

For example, all the beer brewing strains formed a monophyletic group and clustered together in accordance with their application in which they were clearly separated from strains employed in other industries.

These results again demonstrate that beer brewing strains developed extensively through evolutionary adaptation, in which strains were subjected to selective stressors, (*i.e.,* breweries) by serial inoculations in order to obtain spontaneous mutants that allowed for the development of industry-favorable qualities, (*i.e.,* stress tolerance and ethanol productivity) [40].



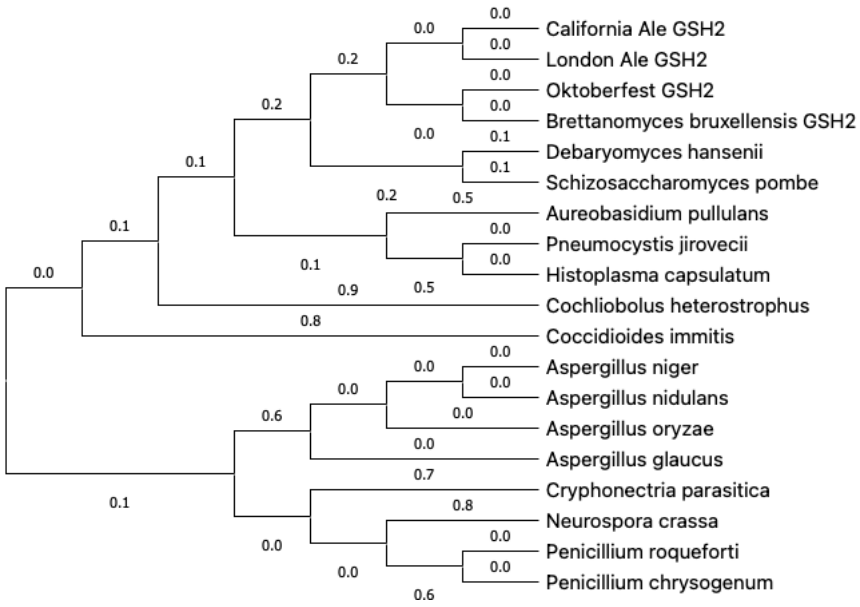


Figure 5. The Extended Phylogenetic Trees

The extended phylogenetic trees included strains used in various industrial applications not associated with beer brewing as well as the experimental stains used solely in beer brewing. As such, beer brewing strains continued to form monophyletic groups and clustered together in accordance with their application. The shortened branch lengths observed in association with beer brewing strains *GSH* pathway further confirmed this analysis as the smaller ratios were proportional to minute changes between their genome sequences.

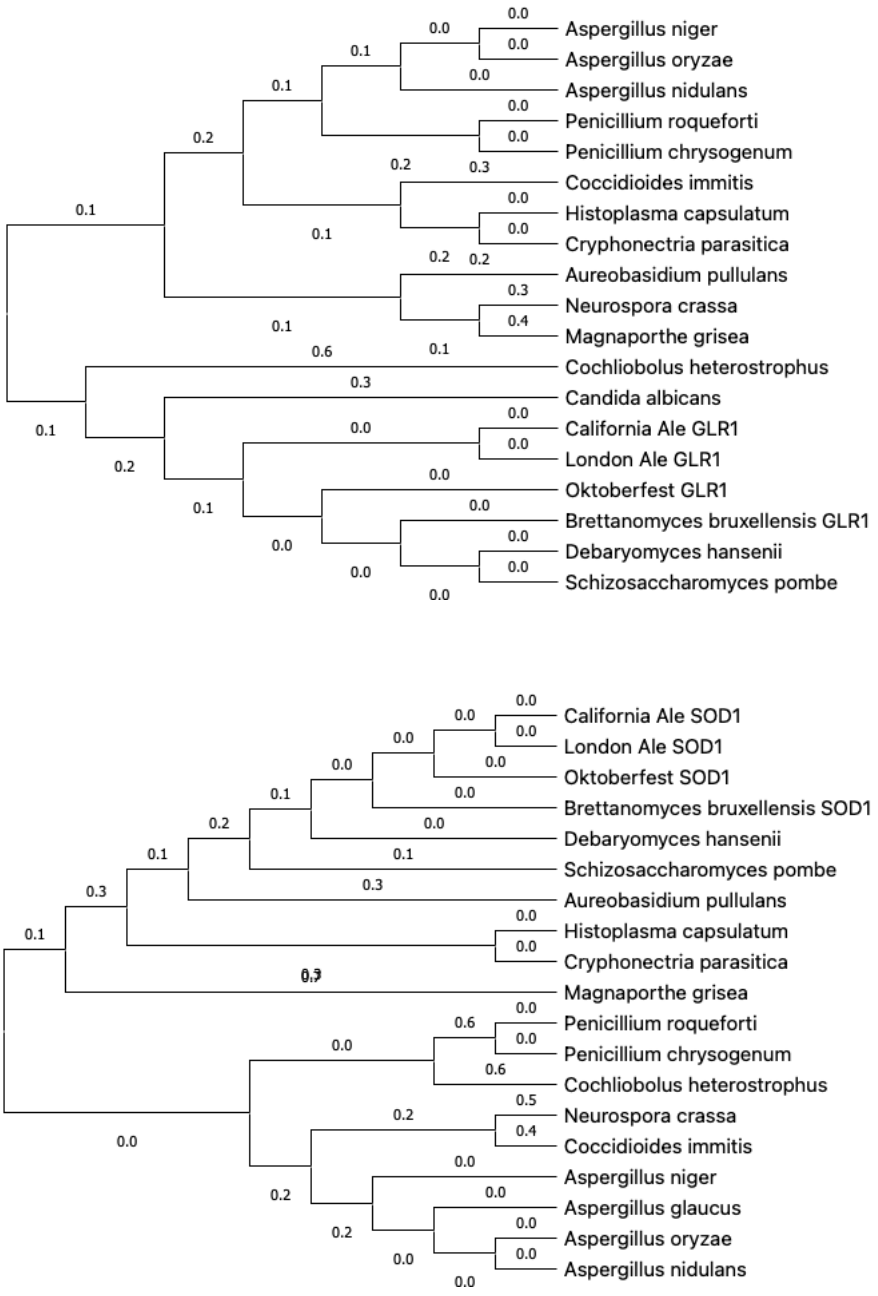


Figure 6. Phylogenetic Tree Construction

Trees constructed from the original and extended datasets were congruent. These findings further confirmed that the evolutionary divergence of industrial yeast is shaped by industrial application and other biological influences such as geographic location.

Nucleotide & Amino Acid Sequences

The proteins that *GSH* pathway genes encode do not function individually but rather within the entire pathway [34]. In fact, our results suggest that natural selection, (*i.e*., beer brewing environment) acted on this group of genes to collectively perform the appropriate biological function [38].

As such, protein coding *GSH* pathway DNA sequences were input into the MEGAX alignment grid and translated into their respective amino acid sequences using the genetic code table to demonstrate the highly conserved nature of both the GSH pathway's nucleotide and amino acid sequences [33].

Due to the highly conserved nature of both the nucleotide and amino acid sequences, it is reasonable to conclude that these four genes/ proteins are crucial for the biological activity of the *GSH* pathway and survivability of yeast strains within industrial beer brewing settings. Previous studies seem to further confirm these findings through investigative experiments using site-directed mutagenesis on *GSH* pathway proteins [23]. Their results showed that *GSH* pathway mutants exhibited undetectable activity when compared with wild-type strains which suggests that all four conserved genes/proteins are critical for enzymatic activity. [23]

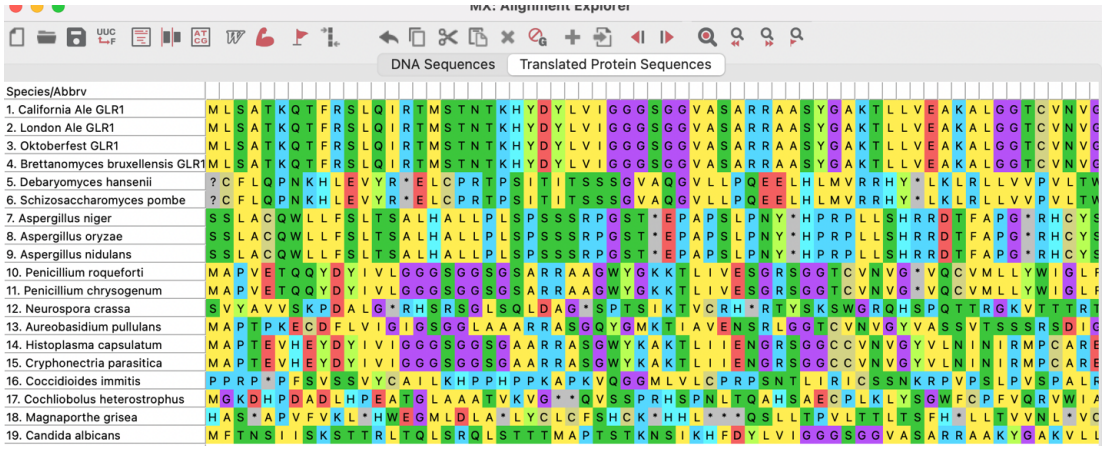


Figure 7. Translated Protein Sequences

The MEGAX alignment grid was employed to translate the GLR1 protein coding DNA sequence into its respective amino acid sequence. Above is shown a partial amino acid sequence alignment.

Conclusions & Limitations of the Study

All phylogenetic studies are limited in some measure with even single gene phylogenies being susceptible to conflict. However, the selection of highly conserved sequences and the removal of problematic regions, (*i.e.*, eliminate regions that cannot be aligned with confidence) has been shown to yield accurate results and produce better trees [34].

One noteworthy observation coming from this investigation is how similar beer brewing yeast are at the genome level, despite their apparent taxonomic differences. Of the four genes sampled, each generated a well-supported tree based on approximate likelihood ratio tests (aLRT) and conserved sequences. This led the investigator to conclude that *GSH* pathway genes are suitable candidates for predicting phylogenetic relationships amongst industrial strains due to their highly conserved nature and metabolic importance.

By providing this information, oxidative stress responses and fermentation performance may be predicted, assessed, and potentially selected for, for thousands of other industrial beer yeasts strains used today. These results can be used as a template or reference chart which will enable brewers to focus on desirable metabolic genes that contribute to improved and enhanced beer products.

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APPENDICES

Table 1. London Ale, California Ale, Oktoberfest, and *Brettanomyces bruxellensis* Primers

GSH 1 Forward Primer (YJL101C)	**5'-GAA GAA TAA AAT GGG ACT C-3'**	25 nmole DNA Oligo	Standard Desalting
GSH1 Reverse Primer (YJL101C)	**5'-CAA CCG AAG TAA AAG GAG T-3'**	25 nmole DNA Oligo	Standard Desalting
GLR1 Forward Primer (YPL091W)	**5'-CAT ATT AGT TTA CAG AAC TTT-3'**	25 nmole DNA Oligo	Standard Desalting
GLR1 Reverse Primer (YPL091W)	**5'-CGT CTA GTT TCG TTG CTT CA-3'**	25 nmole DNA Oligo	Standard Desalting
SOD1 Forward Primer (YJR104C)	**5'-TCG CGC AAA CAA ATA AAA CA-3'**	25 nmole DNA Oligo	Standard Desalting
SOD1 Reverse Primer (YJR104C)	**5'-AAC ATT AGT TGG TTA GAC C-3'**	25 nmole DNA Oligo	Standard Desalting
GSH 2 Forward Primer (YOL049W)	**5'-ATA GGA AGA AAG CAC TAC TCC-3'**	25 nmole DNA Oligo	Standard Desalting
GSH 2 Reverse Primer (YOL049W)	**5'-CAT GTA CAC CTA GTA AAG AA-3'**	25 nmole DNA Oligo	Standard Desalting

Brettanomyces bruxellensis Primers

Separate primers were designed to bind to *Brettanomyces bruxellensis* DNA due to the continued presentation of primer dimers or absence of product when conducted with Strawberry Ale primers. An NCBI BLAST search was conducted for *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to identify similar sequences between the genomes' GSH pathway. Our genes of interest: GSH1, GSH2, GLR1 were keyed out accordingly under the GenBank tab, *Brettanomyces bruxellensis* AWRI1499 AWRI1499_contig525_scaffold5, whole genome shotgun sequence. Each sequence was copied and pasted in FASTA format to yeastgenome.org for primer design. PCR primers were designed using the Web Primer tool, which selects optimal primer pairs based on the uniqueness of each sequence, (multiple primer sets were designed to bind to GSH1 region). SOD1 primers were not designed for *Brettanomyces bruxellensis* because the Strawberry Ale primers were compatible with the *Brettanomyces bruxellensis* SOD1 region.

Table 2. *Brettanomyces bruxellensis* Primers

GSH 1 Forward Primer (YJL101C)	**5'- ATG GGT TTA CTT TCT CAG GG-3' Primer (1)** **5'- ATG GGT TTA CTT TCT CAG GG-3' Primer (2)**	25 nmole DNA Oligo	Standard Desalting
GSH1 Reverse Primer (YJL101C)	**5'- TTA TCC TTG CCA TGT TCT CC-3' Primer (1)** **5'- ATG GGT TTA CTT TCT CAG GG-3' Primer (2)**	25 nmole DNA Oligo	Standard Desalting
GLR1 Forward Primer (YPL091W)	**5'-GCT TAG AGC AAT GAA AAC ACA-3'**	25 nmole DNA Oligo	Standard Desalting
GLR1 Reverse Primer (YPL091W)	**5'-TTG TGA CTA ACT CCT CAG CA-3'**	25 nmole DNA Oligo	Standard Desalting
SOD1 Forward Primer (YJR104C)	**5'-TCG CGC AAA CAA ATA AAA CA-3'**	25 nmole DNA Oligo	Standard Desalting
SOD1 Reverse Primer (YJR104C)	**5'-AAC ATT AGT TGG TTA GAC C-3'**	25 nmole DNA Oligo	Standard Desalting
GSH 2 Forward Primer (YOL049W)	**5'-AGG TCC AAA AGA ATT TCC AC-3'**	25 nmole DNA Oligo	Standard Desalting
GSH 2 Reverse Primer (YOL049W)	**5'-GCC ATT ACG AAG AAG GAC AT-3'**	25 nmole DNA Oligo	Standard Desalting

Table 3. ITS Primers

ITS1F Forward Primer	**5'-CTTGGTCATTTAGAGGAAGTAA-3'**	25 nmole DNA Oligo	Standard Desalting
ITS2 Reverse Primer	**5'- GCTGCGTTCTTCATCGATGC-3'**	25 nmole DNA Oligo	Standard Desalting

CLUSTAL 2.1 GSH1 multiple sequence alignment

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London          ATGGGACTCTTAGCTTTGGGCACGCCTTTGCAGTGGTTTGAGTCTAGGACG
TACAATGAA
California      ATGGGACTCTTAGCTTTGGGCACGCCTTTGCAGTGGTTTGAGTCTAGGACG
TACAATGAA
Oktoberfest     ATGGGACTCTTAGCTTTGGGCACGCCTTTGCAGTGGTTTGAGTCTAGGACG
TACAATGAA
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London          CACATAAGGGATGAAGGTATCGAGCAGTTGTTGTATATTTTCCAAGCTGCT
GGTAAAAGA
California      CACATAAGGGATGAAGGTATCGAGCAGTTGTTGTATATTTTCCAAGCTGCT
GGTAAAAGA
Oktoberfest     CACATAAGGGATGAAGGTATCGAGCAGTTGTTGTATATTTTCCAAGCTGCT
GGTAAAAGA
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London          GACAATGACCCTCTTTTTTGGGGAGACGAGCTTGAGTACATGGTTGTAGAT
TTTGATGAT
California      GACAATGACCCTCTTTTTTGGGGAGACGAGCTTGAGTACATGGTTGTAGAT
TTTGATGAT
Oktoberfest     GACAATGACCCTCTTTTTTGGGGAGACGAGCTTGAGTACATGGTTGTAGAT
TTTGATGAT
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London          AAGGAGAGAAATTCTATGCTCGACGTTTGCCATGACAAGATACTCACTGAG
CTTAATATG
California      AAGGAGAGAAATTCTATGCTCGACGTTTGCCATGACAAGATACTCACTGAG
CTTAATATG
Oktoberfest     AAGGAGAGAAATTCTATGCTCGACGTTTGCCATGACAAGATACTCACTGAG
CTTAATATG
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London          GAGGATTCGTCCCTTTGTGAGGCTAACGATGTGAGTTTTCACCCTGAGTAT
GGCCGGTAT
California      GAGGATTCGTCCCTTTGTGAGGCTAACGATGTGAGTTTTCACCCTGAGTAT
GGCCGGTAT
Oktoberfest     GAGGATTCGTCCCTTTGTGAGGCTAACGATGTGAGTTTTCACCCTGAGTAT
GGCCGGTAT
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London          ATGTTAGAGGCAACACCAGCTTCTCCATATTTGAATTACGTGGGTAGTTAC
GTTGAGGTT
California      ATGTTAGAGGCAACACCAGCTTCTCCATATTTGAATTACGTGGGTAGTTAC
GTTGAGGTT
Oktoberfest     ATGTTAGAGGCAACACCAGCTTCTCCATATTTGAATTACGTGGGTAGTTAC
GTTGAGGTT
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London              AACATGCAAAAAAGACGTGCCATTGCAGAATATAAGCTATCTGAATATGCG
AGACAAGAT
California          AACATGCAAAAAAGACGTGCCATTGCAGAATATAAGCTATCTGAATATGCG
AGACAAGAT
Oktoberfest         AACATGCAAAAAAGACGTGCCATTGCAGAATATAAGCTATCTGAATATGCG
AGACAAGAT
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London              AGTAAAAATAACTTGCATGTGGGCTCCAGGTCTGTCCCTTTGACGCTGACT
GTCTTCCCG
California          AGTAAAAATAACTTGCATGTGGGCTCCAGGTCTGTCCCTTTGACGCTGACT
GTCTTCCCG
Oktoberfest         AGTAAAAATAACTTGCATGTGGGCTCCAGGTCTGTCCCTTTGACGCTGACT
GTCTTCCCG
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London              AGGATGGGATGCCCCGACTTTATTAACATTAAGGATCCGTGGAATCATAAA
AATGCCGCT
California          AGGATGGGATGCCCCGACTTTATTAACATTAAGGATCCGTGGAATCATAAA
AATGCCGCT
Oktoberfest         AGGATGGGATGCCCCGACTTTATTAACATTAAGGATCCGTGGAATCATAAA
AATGCCGCT
                    **************************************************
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London              TCCAGGTCTCTGTTTTTACCCGATGAAGTCATTAACAGACATGTCAGGTTT
CCTAACTTG
California          TCCAGGTCTCTGTTTTTACCCGATGAAGTCATTAACAGACATGTCAGGTTT
CCTAACTTG
Oktoberfest         TCCAGGTCTCTGTTTTTACCCGATGAAGTCATTAACAGACATGTCAGGTTT
CCTAACTTG
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London              ACAGCATCCATCAGGACCAGGCGTGGTGAAAAAGTTTGCATGAATGTTCCC
ATGTATAAA
California          ACAGCATCCATCAGGACCAGGCGTGGTGAAAAAGTTTGCATGAATGTTCCC
ATGTATAAA
Oktoberfest         ACAGCATCCATCAGGACCAGGCGTGGTGAAAAAGTTTGCATGAATGTTCCC
ATGTATAAA
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London              GATATAGCTACTCCAGAAACGGATGACTCCATCTACGATCGAGATTGGTTT
TTACCAGAA
California          GATATAGCTACTCCAGAAACGGATGACTCCATCTACGATCGAGATTGGTTT
TTACCAGAA
Oktoberfest         GATATAGCTACTCCAGAAACGGATGACTCCATCTACGATCGAGATTGGTTT
TTACCAGAA
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London            GACAAAGAGGCGAAACTGGCTTCCAAACCGGGTTTCATTTATATGGATTCC
ATGGGTTTT
California        GACAAAGAGGCGAAACTGGCTTCCAAACCGGGTTTCATTTATATGGATTCC
ATGGGTTTT
Oktoberfest       GACAAAGAGGCGAAACTGGCTTCCAAACCGGGTTTCATTTATATGGATTCC
ATGGGTTTT
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London            GGCATGGGCTGTTCGTGCTTACAAGTGACCTTTCAGGCACCCAATATCAAC
AAGGCACGT
California        GGCATGGGCTGTTCGTGCTTACAAGTGACCTTTCAGGCACCCAATATCAAC
AAGGCACGT
Oktoberfest       GGCATGGGCTGTTCGTGCTTACAAGTGACCTTTCAGGCACCCAATATCAAC
AAGGCACGT
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London            TACCTGTACGATGCATTAGTGAATTTTGCACCTATAATGCTAGCCTTCTCT
GCCGCTGCG
California        TACCTGTACGATGCATTAGTGAATTTTGCACCTATAATGCTAGCCTTCTCT
GCCGCTGCG
Oktoberfest       TACCTGTACGAT----------------------------------------
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London            CCTGCTTTTAAAGGTTGGCTAGCCGACCAAGATGTTCGTTGGAATGTGATA
TCTGGTGCG
California        CCTGCTTTTAAAGGTTGGCTAGCCGACCAAGATGTTCGTTGGAATGTGATA
TCTGGTGCG
Oktoberfest       ------------------------------------------------
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London            GTGGACGACCGTACTCCGAAGGAAAGAGGTGTTGCGCCATTACTACCCAAA
TACAACAAG
California        GTGGACGACCGTACTCCGAAGGAAAGAGGTGTTGCGCCATTACTACCCAAA
TACAACAAG
Oktoberfest       ------------------------------------------------
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London            AACGGATTTGGAGGCATTGCCAAAGACGTACAAGATAAAGTCCTTGAAATA
CCAAAGTCA
California        AACGGATTTGGAGGCATTGCCAAAGACGTACAAGATAAAGTCCTTGAAATA
CCAAAGTCA
Oktoberfest       ------------------------------------------------
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London          AGATATAGTTCGGTTGATCTTTTCTTGGGTGGGTCGAAATTTTTCAATAGG
ACTTATAAC
California      AGATATAGTTCGGTTGATCTTTTCTTGGGTGGGTCGAAATTTTTCAATAGG
ACTTATAAC
Oktoberfest     ----------
CGGTTGATCTTTTCTTGGGTGGGTCGAAATTTTTCAATAGGACTTATAAC
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London          GACACAAATGTACCTATTAATGAAAAAGTATTAGGACGACTACTAGAGAAT
GATAAGGCG
California      GACACAAATGTACCTATTAATGAAAAAGTATTAGGACGACTACTAGAGAAT
GATAAGGCG
Oktoberfest     GACACAAATGTACCTATTAATGAAAAAGTATTAGGACGACTACTAGAGAAT
GATAAGGCG
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London          CCACTGGACTATGATCTTGCTAAACATTTTGCGCATCTCTACATAAGAGAT
CCAGTATCT
California      CCACTGGACTATGATCTTGCTAAACATTTTGCGCATCTCTACATAAGAGAT
CCAGTATCT
Oktoberfest     CCACTGGACTATGATCTTGCTAAACATTTTGCGCATCTCTACATAAGAGAT
CCAGTATCT
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London          ACATTCGAAGAACTGTTGAATCAGGACAACAAAACGTCTTCAAATCACTTT
GAAAACATC
California      ACATTCGAAGAACTGTTGAATCAGGACAACAAAACGTCTTCAAATCACTTT
GAAAACATC
Oktoberfest     ACATTCGAAGAACTGTTGAATCAGGACAACAAAACGTCTTCAAATCACTTT
GAAAACATC
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London          CAAAGTACAAATTGGCAGACATTACGTTTTAAACCCCCCACACAACAAGCA
ACCCCGGAC
California      CAAAGTACAAATTGGCAGACATTACGTTTTAAACCCCCCACACAACAAGCA
ACCCCGGAC
Oktoberfest     CAAAGTACAAATTGGCAGACATTACGTTTTAAACCCCCCACACAACAAGCA
ACCCCGGAC
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London          AAAAAGGATTCTCCTGGTTGGAGAGTGGAATTCAGACCATTTGAAGTGCAA
CTATTAGAT
California      AAAAAGGATTCTCCTGGTTGGAGAGTGGAATTCAGACCATTTGAAGTGCAA
CTATTAGAT
Oktoberfest     AAAAAGGATTCTCCTGGTTGGAGAGTGGAATTCAGACCATTTGAAGTGCAA
CTATTAGAT
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London              TTTGAGAACGCTGCGTATTCCGTGCTCATATACTTGATTGTCGATAGCATT
TTGACCTTT
California          TTTGAGAACGCTGCGTATTCCGTGCTCATATACTTGATTGTCGATAGCATT
TTGACCTTT
Oktoberfest         TTTGAGAACGCTGCGTATTCCGTGCTCATATACTTGATTGTCGATAGCATT
TTGACCTTT
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London              TCCGATAATATTAACGCATATATTCATATGTCCAAAGTATGGGAAAATATG
AAGATAGCC
California          TCCGATAATATTAACGCATATATTCATATGTCCAAAGTATGGGAAAATATG
AAGATAGCC
Oktoberfest         TCCGATAATATTAACGCATATATTCATATGTCCAAAGTATGGGAAAATATG
AAGATAGCC
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London              CATCACAGAGATGCTATCCTATTTGAAAAATTTCATTGGAAAAAATCATTT
CGCAACGAC
California          CATCACAGAGATGCTATCCTATTTGAAAAATTTCATTGGAAAAAATCATTT
CGCAACGAC
Oktoberfest         CATCACAGAGATGCTATCCTATTTGAAAAATTTCATTGGAAAAAATCATTT
CGCAACGAC
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London              ACCGATGTGGAAACTGAAGATTATTCTATAAGCGAGATTTTCCATAATCCA
GAGAATGGT
California          ACCGATGTGGAAACTGAAGATTATTCTATAAGCGAGATTTTCCATAATCCA
GAGAATGGT
Oktoberfest         ACCGATGTGGAAACTGAAGATTATTCTATAAGCGAGATTTTCCATAATCCA
GAGAATGGT
                    **************************************************
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London              ATATTTCCTCAATTTGTTACGCCAATCCTATGCCAAAAAGGGTTTGTAACC
AAAGATTGG
California          ATATTTCCTCAATTTGTTACGCCAATCCTATGCCAAAAAGGGTTTGTAACC
AAAGATTGG
Oktoberfest         ATATTTCCTCAATTTGTTACGCCAATCCTATGCCAAAAAGGGTTTGTAACC
AAAGATTGG
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London              AAAGAATTAAAGCATTCTTCCAAACACGAGAGACTATACTATTATTTAAAG
CTAATTTCT
California          AAAGAATTAAAGCATTCTTCCAAACACGAGAGACTATACTATTATTTAAAG
CTAATTTCT
Oktoberfest         AAAGAATTAAAGCATTCTTCCAAACACGAGAGACTATACTATTATTTAAAG
CTAATTTCT
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London            GATAGAGCAAGCGGTGAATTGCCAACAACAGCAAAATTCTTTAGAAATTTT
GTACTACAA
California        GATAGAGCAAGCGGTGAATTGCCAACAACAGCAAAATTCTTTAGAAATTTT
GTACTACAA
Oktoberfest       GATAGAGCAAGCGGTGAATTGCCAACAACAGCAAAATTCTTTAGAAATTTT
GTACTACAA
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London            CATCCAGATTACAAACATGATTCAAAAATTTCAAAGTCGATCAATTATGAT
TTGCTTTCT
California        CATCCAGATTACAAACATGATTCAAAAATTTCAAAGTCGATCAATTATGAT
TTGCTTTCT
Oktoberfest       CATCCAGATTACAAACATGATTCAAAAATTTCAAAGTCGATCAATTATGAT
TTGCTTTCT
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London            ACGTGTGATAGACTTACCCATTTAGACGATTCAAAAGGTGAATTGACATCC
TTTTTAGGA
California        ACGTGTGATAGACTTACCCATTTAGACGATTCAAAAGGTGAATTGACATCC
TTTTTAGGA
Oktoberfest       ACGTGTGATAGACTTACCCATTTAGACGATTCAAAAGGTGTTTTAGGATAT
TTAG-----
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London            GCTGAAATTGCAGAATATGTAAAAAAAAAATAAGCCTTCAATAGAAAGCAAA
TGTTAA
California        GCTGAAATTGCAGAATATGTAAAAAAAAAATAAGCCTTCAATAGAAAGCAAA
TGTTAA
Oktoberfest       --------------------------------------------------
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CLUSTAL 2.1 GSH2 multiple sequence alignment

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London              ATGGCACACTATCCACCTTCCAAGGATCAATTGAATGAATTGATCCAGG
AAGTTAACCAA
California          ATGGCACACTATCCACCTTCCAAGGATCAATTGAATGAATTGATCCAGG
AAGTTAACCAA
Oktoberfest         ATGGCACACTATCCACCTTCCAAGGATCAATTGAATGAATTGATCCAGG
AAGTTAACCAA
Brettanomyces       ATNGCCCANTANCCGCCTTCCAACGANCAATTGAATGAATTCATCCAGG
AAGTNAACCAA
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London              TCGGTATCACTAATGGATTATCCATGTATCCTCCTAAATTCGAGGAGAA
CCCATCAAAT
California          TGGGCTATCACTAATGGATTATCCATGTATCCTCCTAAATTCGAGGAGA
ACCCATCAAAT
Oktoberfest         TGGGCTATCACTAATGGATTATCCATGTATCCTCCTAAATTCGAGGAGA
ACCCATCAAAT
Brettanomyces       TCGGNTATCAGTATTGNATTATCCANGTTTCCTCCTAAATTCGNGGAGA
ACCCATCAAAT
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London              GCATCGGTGTCACCAGTAACTATCTATCCAACCCCAATTCCTAGGAAAT
GTTTTGATGAG
California          GCATCGGTGTCACCAGTAACTATCTATCCAACCCCAATTCCTAGGAAAT
GTTTTGATGAG
Oktoberfest         GCATCGGTGTCACCAGTAACTATCTATCCAACCCCAATTCCTAGGAAAT
GTTTTGATGAG
Brettanomyces       NCAGCGGTGTCAGCANTAACTATCTATCCAACCCCNATTCCTAGGAAAT
GTTTTGATGAG
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London              GCCGTTCAAATACAACCGGTATTCAATGAATTATACGCCCGTATTACCC
AAGATATGGCC
California          GCCGTTCAAATACAACCGGTATTCAATGAATTATACGCCCGTATTACCC
AAGATATGGCC
Oktoberfest         GCCGTTCAAATACAACCGGTATTCAATGAATTATACGCCCGTATTACCC
AAGATATGGCC
Brettanomyces       GCCGTTCAAATACAACCGGTATTCAATGAATTATACGCCCGTATTACCC
AAGATATGGCC
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London              CAACCTGATTCTTATTTACATAAAACAACTGAAGCGTTAGCTCTATCAG
ATTCCGAGTTT
California          CAACCTGATTCTTATTTACATAAAACAACTGAAGCGTTAGCTCTATCAG
ATTCCGAGTTT
Oktoberfest         CAACCTGATTCTTATTTACATAAAACAACTGAAGCGTTAGCTCTATCAG
ATTCCGAGTTT
Brettanomyces       CAACCTGATTCTTATTTACATAAAACAACTGAAGCGTTAGCTCTATCAG
ATTCCGAGTTT
                    **************************************************
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London              ACTGGAAAACTGTGGTCTCTATACCTTGCTACCTTAAAATCTGCACAGT
ACAAAAAGCAG
California          ACTGGAAAACTGTGGTCTCTATACCTTGCTACCTTAAAATCTGCACAGT
ACAAAAAGCAG
Oktoberfest         ACTGGAAAACTGTGGTCTCTATACCTTGCTACCTTAAAATCTGCACAGT
ACAAAAAGCAG
Brettanomyces       ACTGGAAAACTGTGGTCTCTATACCTTGCTACCTTAAAATCTGCACAGT
ACAAAAAGCAG
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London              AATTTTAGGCTAGGTATATTTAGATCAGATTATTTGATTGATAAGAAAA
AGGGTACTGAA
California          AATTTTAGGCTAGGTATATTTAGATCAGATTATTTGATTGATAAGAAAA
AGGGTACTGAA
Oktoberfest         AATTTTAGGCTAGGTATATTTAGATCAGATTATTTGATTGATAAGAAAA
AGGGTACTGAA
Brettanomyces       AATTTTAGGCTAGGTATATTTAGATCAGATTATTTGATTGATAAGAAAA
AGGGTACTGAA
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London              CAGATTAAGCAAGTCGAGTTTAATACAGTGTCAGTGTCATTTGCAGGCC
TTAGCGAGAAA
California          CAGATTAAGCAAGTCGAGTTTAATACAGTGTCAGTGTCATTTGCAGGCC
TTAGCGAGAAA
Oktoberfest         CAGATTAAGCAAGTCGAGTTTAATACAGTGTCAGTGTCATTTGCAGGCC
TTAGCGAGAAA
Brettanomyces       CAGATTAAGCAAGTCGAGTTTAATACAGTGTCAGTGTCATTTGCAGGCC
TTAGCGAG---
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London              GTTGATAGATTGCACTCTTATTTAAATAGGGCAAACAAGTACGATCCTA
AAGGACCAATT
California          GTTGATAGATTGCACTCTTATTTAAATAGGGCAAACAAGTACGATCCTA
AAGGACCAATT
Oktoberfest         GTTGATAGATTGCACTCTTATTTAAATAGGGCAAACAAGTACGATCCTA
AAGGACCAATT
Brettanomyces       --------------------------------------------------
---------TT

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London              TATAATGATCAAAATATGGTCATTTCTGATTCAGGATACCTTTTGTCTA
AGGCATTGGCC
California          TATAATGATCAAAATATGGTCATTTCTGATTCAGGATACCTTTTGTCTA
AGGCATTGGCC
Oktoberfest         TATAATGATCAAAATATGGTCATTTCTGATTCAGGATACCTTTTGTCTA
AGGCATTGGCC
Brettanomyces       TATAATGATCAAAATATGGTCATTTCTGATTCAGGATACCTTTTGTCTA
AGGCATTGGCC
                    **************************************************
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London              AAAGCTGTGGAATCGTATAAGTCACAACAAAGTTCTTCTACAACTAGTG
ATCCTATTGTC
California          AAAGCTGTGGAATCGTATAAGTCACAACAAAGTTCTTCTACAACTAGTG
ATCCTATTGTC
Oktoberfest         AAAGCTGTGGAATCGTATAAGTCACAACAAAGTTCTTCTACAACTAGTG
ATCCTATTGTC
Brettanomyces       AAAGCTGTGGAATCGTATAAGTCACAACAAAGTTCTTCTACAACTAGTG
ATCCTATTGTC
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London              GCATTCATTGTGCAAAGAAACGAGAGAAATGTGTTTGATCAAAAGGTCT
TGGAATTGAAT
California          GCATTCATTGTGCAAAGAAACGAGAGAAATGTGTTTGATCAAAAGGTCT
TGGAATTGAAT
Oktoberfest         GCATTCATTGTGCAAAGAAACGAGAGAAATGTGTTTGATCAAAAGGTCT
TGGAATTGAAT
Brettanomyces       GCATTCATTGTGCAAAGAAACGAGAGAAATGTGTTTGATCAAAAGGTCT
TGGAATTGAAT
                    **************************************************
***********

London              CTGTTGGAAAAATTCGGTACCAAATCTGTTAGGTTGACGTTTGATGATG
TTAACGATAAA
California          CTGTTGGAAAAATTCGGTACCAAATCTGTTAGGTTGACGTTTGATGATG
TTAACGATAAA
Oktoberfest         CTGTTGGAAAAATTCGGTACCAAATCTGTTAGGTTGACGTTTGATGATG
T----------
Brettanomyces       CTGTTGGAAAAATTCGGTACCAAATCTGTTAGGTTGACGTTTGATGATG
TTAACGATAAA
                    **************************************************
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London              TTGTTCATTGATGATAAAACGGGAAAGCTTTTCATTAGGGACACAGAGC
AGGAAATAGCG
California          TTGTTCATTGATGATAAAACGGGAAAGCTTTTCATTAGGGACACAGAGC
AGGAAATAGCG
Oktoberfest         --------------------------------------------------
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Brettanomyces       TTGTTCATTGATGATAAAACGGGAAAGCTTTTCATTAGGGACACAGAGC
AGGAAATAGCG



London              GTGGTTTATTACAGAACGGGTTACACAACCACTGATTACACGTCCGAAA
AGGACTGGGAG
California          GTGGTTTATTACAGAACGGGTTACACAACCACTGATTACACGTCCGAAA
AGGACTGGGAG
Oktoberfest         GTGGTTTATTACAGAACGGGTTACACAACCACTGATTACACGTCCGAAA
AGGACTGGGAG
Brettanomyces       GTGGTTTATTACAGAACGGGTTACACAACCACTGATTACACGTCCGAAA
AGGACTGGGAG
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***********

London              GCAAGACTATTCCTCGAAAAAAGTTTCGCAATAAAGGCCCCAGATTTAC
TCACTCAATTA
California          GCAAGACTATTCCTCGAAAAAAGTTTCGCAATAAAGGCCCCAGATTTAC
TCACTCAATTA
Oktoberfest         GCAAGACTATTCCTCGAAAAAAGTTTCGCAATAAAGGCCCCAGATTTAC
TCACTCAATTA
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Brettanomyces      GCAAGACTATTCCTCGAAAAAAGTTTCGCAATAAAGGCCCCAGATTTAC
TCACTCAATTA
                   **************************************************
**********

London             TCTGGCTCCAAGAAAATTCAGCAATTGTTGACAGATGAGGGCGTATTAG
GTAAATACATC
California         TCTGGCTCCAAGAAAATTCAGCAATTGTTGACAGATGAGGGCGTATTAG
GTAAATACATC
Oktoberfest        TCTGGCTCCAAGAAAATTCAGCAATTGTTGACAGATGAGGGCGTATTAG
GTAAATACATC
Brettanomyces      TCTGGCTCCAAGAAAATTCAGCAATTGTTGACAGATGAGGGCGTATTAG
GTAAATACATC
                   **************************************************
**********

London             TCCGATGCTGAGAAAAAGAGTAGTTTGTTAAAAACTTTTGTCAAAATAT
ATCCCTTGGAT
California         TCCGATGCTGAGAAAAAGAGTAGTTTGTTAAAAACTTTTGTCAAAATAT
ATCCCTTGGAT
Oktoberfest        TCCGATGCTGAGAAAAAGAGTAGTTTGTTAAAAACTTTTGTCAAAATAT
ATCCCTTGGAT
Brettanomyces      TCCGATGCTGAGAAAAAGAGTAGTTTGTTAAAAACTTTTGTCAAAATAT
ATCCCTTGGAT
                   **************************************************
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London             GATACGAAGCTTGGCAGGGAAGGCAAGAGGCTGGCATTAAGTGAGCCCT
CTAAATACGTG
California         GATACGAAGCTTGGCAGGGAAGGCAAGAGGCTGGCATTAAGTGAGCCCT
CTAAATACGTG
Oktoberfest        GATACGAAGCTTGGCAGGGAAGGCAAGAGGCTGGCATTAAGTGAGCCCT
CTAAATACGTG
Brettanomyces      GA------------------
AGGCAAGAGGCTGGCATTAAGTGAGCCCTCTAAATACGTG
                   **                  ******************************
**********

London             TTAAAACCACAGCGGGAAGGTGGCGGAAACAATGTTTATAAAGAAAATA
TTCCTAATTTT
California         TTAAAACCACAGCGGGAAGGTGGCGGAAACAATGTTTATAAAGAAAATA
TTCCTAATTTT
Oktoberfest        TTAAAACCACAGCGGGAAGGTGGCGGAAACAATGTTTATAAAGAAAATA
TTCCTAATTTT
Brettanomyces      TTAAAACCACAGCGGGAAGGTGGCGGAAACAATGTTTATAAAGAAAATA
TTCCTAATTTT
                   **************************************************
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London             TTGAAAGGTATCGAAGAACGTCACTGGGATGCATATATTCTCATGGAGT
TGATTGAACCA
California         TTGAAAGGTATCGAAGAACGTCACTGGGATGCATATATTCTCATGGAGT
TGATTGAACCA
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Oktoberfest         TTGAAAGGTATCGAAGAACGTCACTGGGATGCATATATTCTCATGGAGT
TGATTGAACCA
Brettanomyces       TTGAAAGGTATCGAAGAACGTCACTGGGATGCATATATTCTCATGGAGT
TGATTGAACCA
                    **************************************************
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London              GAGTTGAATGAAAATAATATTATATTACGTGATAACAAATCTTACAACG
AACCAATCATC
California          GAGTTGAATGAAAATAATATTATATTACGTGATAACAAATCTTACAACG
AACCAATCATC
Oktoberfest         GAGTTGAATGAAAATAATATTATATTACGTGATAACAAATCTTACAACG
AACCAATCATC
Brettanomyces       GAGTTGAATGAAAATAATATTATATTACGTGATAACAAATCTTACAACG
AACCAATCATC
                    **********************************************
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London              AGTGAACTAGGAATTTATGGTTGCGTTCTATTTAACGACGAGCAAGTTT
TATCGAACGAA
California          AGTGAACTAGGAATTTATGGTTGCGTTCTATTTAACGACGAGCAAGTTT
TATCGAACGAA
Oktoberfest         AGTGAACTAGGAATTTATGGTTGCGTTCTATTTAACGACGAGCAAGTTT
TATCGAACGAA
Brettanomyces       AGTGAACTAG---TTTAAGGCCTAGGTTTTTTAAAAGGGGCCC---
CTTTACCAAACCCC
                    **********   **** **    * * * ** **
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London              TTTAGTGGCTCATTACTAAGATCCAAATTTAATACTTCAAATGAAGGTG
GAGTGGCGGCA
California          TTTAGTGGCTCATTACTAAGATCCAAATTTAATACTTCAAATGAAGGTG
GAGTGGCGGCA
Oktoberfest         TTTAGTGGCTCATTACTAAGATCCAAATTTAATACTTCAAATGAAGGTG
GAGTGGCGGCA
Brettanomyces       CCGGGGGTTTTCCCCGAGAGATTTTGAAAAGGTCTTGGAATTGAATCTG
TTGGAAAAATT
                        * *  *        ****    *     *  *  **
****  **  *

London              GGATTCGGATGTTTGGACAGTATTATTCTTTACTAG-------------
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California          GGATTCGGATGTTTGGACAGTATTATTCTTTACTAG-------------
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Oktoberfest         GGATTCGGATGTTTGGACAGTATTATTCTTTACTAGCCCAAATCCCCAA
ACCTTGGGGGT
Brettanomyces       CGGTACCAAATCTGTTAGGTTGACGTTTGATGATGT-------------
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*  *   *   *   *    **   *  *

London              ----------------------------------------
California          ----------------------------------------
Oktoberfest         CAAAAAATTTTGGGCCGGCGCGCTTTAAAATTTGTGTCCC
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Brettanomyces      ----------------------------------------
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CLUSTAL 2.1 GLR1 multiple sequence alignment

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London             ATGCTTTCTGCAACCAAACAAACATTTAGAAGTCTACAGATAAGAACTA
TGTCCACGAAC
California         ATGCTTTCTGCAACCAAACAAACATTTAGAAGTCTACAGATAAGAACTA
TGTCCACGAAC
Oktoberfest        ATGCTTTCTGCAACCAAACAAACATTTAGAAGTCTACAGATAAGAACTA
TGTCCACGAAC
Brettanomyces      ATCCTATCGGCCACTAAACAAACTTTGAGNAGTCTACAGATAAGAACTA
TGTCCACGAAC
                   **************************************************
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London             ACCAAGCATTACGATTACCTCGTCATCGGGGGTGGCTCAGGGGGTGTTG
CTTCCGCAAGA
California         ACCAAGCATTACGATTACCTCGTCATCGGGGGTGGCTCAGGGGGTGTTG
CTTCCGCAAGA
Oktoberfest        ACCAAGCATTACGATTACCTCGTCATCGGGGGTGGCTCAGGGGGTGTTG
CTTCCGCAAGA
Brettanomyces      NCCAAGCATTACGATTACGTCGTCATCGGGGGTCGCACACGGGGTGTTG
CTTCCACAAGA
                   **************************************************
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London             AGAGCTGCATCTTATGGTGCGAAGACATTACTAGTTGAAGCTAAGGCTC
TTGGTGGTACC
California         AGAGCTGCATCTTATGGTGCGAAGACATTACTAGTTGAAGCTAAGGCTC
TTGGTGGTACC
Oktoberfest        AGAGCTGCATCTTATGGTGCGAAGACATTACTAGTTGAAGCTAAGGCTC
TTGGTGGTACC
Brettanomyces      CGAGCTGCATCTTATGGTGCGAAGACATTACTAGTTGAAGCTAAGGCTC
TTGGTGGTACC
                   **************************************************
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London             TGTGTTAACGTGGGTTGTGTTCCGAAGAAAGTCATGTGGTATGCTTCTG
ACCTCGCTACT
California         TGTGTTAACGTGGGTTGTGTTCCGAAGAAAGTCATGTGGTATGCTTCTG
ACCTCGCTACT
Oktoberfest        TGTGTTAACGTGGGTTGTGTTCCGAAGAAAGTCATGTGGTATGCTTCTG
ACCTCGCTACT
Brettanomyces      TGTGTTAACGTGGGTTGTGTTCCGAAGAAAGTCATGTGGTATGCTTCTG
ACCTCGCTACT
                   **************************************************
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London             AGAGTATCCCATGCAAATGAATATGGATTATATCAGAATCTTCCATTAG
ATAAAGAGCAT
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California          AGAGTATCCCATGCAAATGAATATGGATTATATCAGAATCTTCCATTAG
ATAAAGAGCAT
Oktoberfest         AGAGTATCCCATGCAAATGAATATGGATTATATCAGAATCTTCCATTAG
ATAAAGAGCAT
Brettanomyces       AGAGTATCCCATGCAAATGAATATGGATTATATCAGAATCTTCCATTAG
ATAAAGAGCAT
                    **************************************************
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London              TTGACTTTTAATTGGCCAGAATTTAAGCAGAAAAGGGATGCTTATGTCC
ATAGGTTGAAC
California          TTGACTTTTAATTGGCCAGAATTTAAGCAGAAAAGGGATGCTTATGTCC
ATAGGTTGAAC
Oktoberfest         TTGACTTTTAATTGGCCAGAATTTAAGCAGAAAAGGGATGCTTATGTCC
ATAGGTTGAAC
Brettanomyces       TTGACTTTTAATTGGCCAGAATTTAAGCAGAAAAGGGATGCTTATGTCC
ATAGGTTGAAC
                    **************************************************
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London              GGTATATACCAGAAGAATTTAGAAAAAGAAAAAGTGGATGTTGTATTTG
GATGGGCTAGA
California          GGTATATACCAGAAGAATTTAGAAAAAGAAAAAGTGGATGTTGTATTTG
GATGGGCTAGA
Oktoberfest         GGTATATACCAGAAGAATTTAGAAAAAGAAAAAGTGGATGTTGTATTTG
GATGGGCTAGA
Brettanomyces       GGTATATACCAGAAGAATTTAGAAAAAGAAAAAGTGGATGTTGTATTTG
GATGGGCTAGA
                    **************************************************
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London              TTCAATAAGGACGGTAATGTTGAAGTTCAGAAAAGGGATAATACTACTG
AAGTTTACTCC
California          TTCAATAAGGACGGTAATGTTGAAGTTCAGAAAAGGGATAATACTACTG
AAGTTTACTCC
Oktoberfest         TTCAATAAGGACGGTAATGTTGAAGTTCAGAAAAGGGATAATACTACTG
AAGTTTACTCC
Brettanomyces       TTCAATAAGGACGGTAATGTTGAAGTTCAGAAAAGGGATAATACTACTG
AAGTTTACTCC
                    **************************************************
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London              GCTAACCATATTTTAGTTGCGACCGGTGGAAAGGCTATTTTCCCCGAAA
ACATTCCAGGT
California          GCTAACCATATTTTAGTTGCGACCGGTGGAAAGGCTATTTTCCCCGAAA
ACATTCCAGGT
Oktoberfest         GCTAACCATATTTTAGTTGCGACCGGTGGAAAGGCTATTTTCCCCGAAA
ACATTCCAGGT
Brettanomyces       GCTAACCATATTTTAGTTGCGACCGGTGGAAAGGCTATTTTCCCCGAAA
ACATTCCAGGT
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London              TTCGAATTAGGTACTGATTCTGATGGGTTCTTTAGATTGGAAGAACAAC
CTAAGAAAGTT
California          TTCGAATTAGGTACTGATTCTGATGGGTTCTTTAGATTGGAAGAACAAC
CTAAGAAAGTT
Oktoberfest         TTCGAATTAGGTACTGATTCTGATGGGTTCTTTAGATTGGAAGAACAAC
CTAAGAAAGTT
Brettanomyces       TTCNAATTAGGTACTGATTCTGATGGGTTCTTTAGATTGGAAGAACAAC
CTAAGAAAGTT
                    **************************************************
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London              GTTGTTGTTGGCGCTGGTTATATTGGTATTGAGCTAGCAGGTGTGTTCC
ATGGGCTGGGA
California          GTTGTTGTTGGCGCTGGTTATATTGGTATTGAGCTAGCAGGTGTGTTCC
ATGGGCTGGGA
Oktoberfest         GTTGTTGTTGGCGCTGGTTATATTGGTATTGAGCTAGCAGGTGTGTTCC
ATGGGCTGGGA
Brettanomyces       GTTGTTGTTGGCGCTGGTTATATTGGTATTGAGCTAGCAGGTGTGTTCC
ATGGGCTGGGA
                    **************************************************
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London              TCCGAAACGCACTTGGTAATTAGAGGTGAAACTGTCTTGAGAAAATTTG
ATGAATGCATC
California          TCCGAAACGCACTTGGTAATTAGAGGTGAAACTGTCTTGAGAAAATTTG
ATGAATGCATC
Oktoberfest         TCCGAAACGCACTTGGTAATTAGAGGTGAAACTGTCTTGAGAAAATTTG
ATGAATGCATC
Brettanomyces       TCCGAAACGCACTTGGTAATTAGAGGTGAAACTGTCTTGAGAAAATTTG
ATGAATGCATC
                    **************************************************
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London              CAGAACACTATTACTGACCATTACGTAAAGGAAGGCATCAACGTTCATA
AACTATCCAAA
California          CAGAACACTATTACTGACCATTACGTAAAGGAAGGCATCAACGTTCATA
AACTATCCAAA
Oktoberfest         CAGAACACTATTACTGACCATTACGTAAAGGAAGGCATCAACGTTCATA
AACTATCCAAA
Brettanomyces       CAGAACACTATTACTGACCATTACGTAAAGGAAGGCATCAACGTTCATA
AACTATCCAAA
                    **************************************************
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London              ATTGTTAAGGTGGAGAAAAATGTAGAAACTGACAAACTGAAAATACATA
TGAATGACTCA
California          ATTGTTAAGGTGGAGAAAAATGTAGAAACTGACAAACTGAAAATACATA
TGAATGACTCA
Oktoberfest         ATTGTTAAGGTGGAGAAAAATGTAGAAACTGACAAACTGAAAATACATA
TGAATGACTCA
Brettanomyces       ATTGTTAAGGTGGAGAAAAATGTAGAAACTGACAAACTGAAAATACATA
TGAATGACTCA
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London              AAGTCCATCGATGACGTTGACGAATTAATTTGGACAATTGGACGTAAAT
CCCATCTAGGT
California          AAGTCCATCGATGACGTTGACGAATTAATTTGGACAATTGGACGTAAAT
CCCATCTAGGT
Oktoberfest         AAGTCCATCGATGACGTTGACGAATTAATTTGGACAATTGGACGTAAAT
CCCATCTAGGT
Brettanomyces       AAGTCCATCGATGACGTTGACGAATTAATTTGGACAATTGGACGTAAAT
CCCATCTA---
                    **************************************************
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London              ATGGGTTCAGAAAATGTAGGTATAAAGCTGAACTCTCATGACCAAATAA
TTGCTGATGAA
California          ATGGGTTCAGAAAATGTAGGTATAAAGCTGAACTCTCATGACCAAATAA
TTGCTGATGAA
Oktoberfest         ATGGGTTCAGAAAATGTAGGTATAAAGCTGAACTCTCATGACCAAATAA
TTGCTGATGAA
Brettanomyces       --------
AGAAAATGTAGGTATAAAGCTNAACTCTCATGACCAAATAATTGCTGATGAA
                            ***************************************
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London              TATCAGAACACCAATGTTCCAAACATTTATTCTCTAGGTGACGTTGTTG
GAAAAGTTGAA
California          TATCAGAACACCAATGTTCCAAACATTTATTCTCTAGGTGACGTTGTTG
GAAAAGTTGAA
Oktoberfest         TATCAGAACACCAATGTTCCAAACATTTATTCTCTAGGTGACGTTGTTG
GAAAAGTTGAA
Brettanomyces       TATCAGAACACCAATGTTCCAAACATTTATTCTCTAGGTGACGTTGTT-
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                    **********************************************


London              TTGACACCTGTCGCTATTGCAGCGGGCAGAAAGCTGTCTAATAGACTGT
TTGGTCCAGAG
California          TTGACACCTGTCGCTATTGCAGCGGGCAGAAAGCTGTCTAATAGACTGT
TTGGTCCAGAG
Oktoberfest         TTGACACCTGTCGCTATTGCAGCGGGCAGAAAGCTGTCTAATAGACTGT
TTGGTCCAGAG
Brettanomyces       ----------------------------------------------------
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London              AAATTCCGTAATGACAAACTAGATTACGAGAACGTCCCCAGCGTAATTT
TCTCACATCCT
California          AAATTCCGTAATGACAAACTAGATTACGAGAACGTCCCCAGCGTAATTT
TCTCACATCCT
Oktoberfest         AAATTCCGTAATGACAAACTAGATTACGAGAACGTCCCCAGCGTAATTT
TCTCACATCCT
Brettanomyces       -----
CCGTAATGACAAACTAGATTACGAGAACGTCCCCAGCGTAATTTTCTCACATCCT
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                                  ******************************************
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London            GAAGCCGGTTCCATTGGTATTTCTGAGAAGGAAGCCATTGAAAAGTACG
GTAAGGAGAAT
California        GAAGCCGGTTCCATTGGTATTTCTGAGAAGGAAGCCATTGAAAAGTACG
GTAAGGAGAAT
Oktoberfest       GAAGCCGGTTCCATTGGTATTTCTGAGAAGGAAGCCATTGAAAAGTACG
GTAAGGAGAAT
Brettanomyces     GAAGCCGGTTCCATTGGTATTTCTGAGAAGGAAGCCATTGAAAAGTACG
GTAAGGAGAAT
                  **************************************************
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London            ATAAAGGTCTACAATTCCAAATTTACCGCCATGTACTATGCTATGTTGA
GTGAGAAATCA
California        ATAAAGGTCTACAATTCCAAATTTACCGCCATGTACTATGCTATGTTGA
GTGAGAAATCA
Oktoberfest       ATAAAGGTCTACAATTCCAAATTTACCGCCATGTACTATGCTATGTTGA
GTGAGAAATCA
Brettanomyces     ATAAAGGTCTACAATTCCAAATTTACCGCCATGTACTATGCTATGTTGA
GTGAGAAATCA
                  **************************************************
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London            CCCACAAGATATAAAATTGTTTGTGCGGGACCAAATGAAAAGGTTGTCG
GTCTGCACATT
California        CCCACAAGATATAAAATTGTTTGTGCGGGACCAAATGAAAAGGTTGTCG
GTCTGCACATT
Oktoberfest       CCCACAAGATATAAAATTGTTTGTGCGGGACCAAATGAAAAGGTTGTCG
GTCTGCACATT
Brettanomyces     CCCACAAGATATAAAATTGTTTGTGCGGGACCAAATGAAAAGGTTGTCG
GTCTGCACATT
                  **************************************************
**********

London            GTTGGTGATTCCTCTGCAGAAATCTTGCAAGGGTTCGGTGTTGCTATAA
AGATGGGTGCC
California        GTTGGTGATTCCTCTGCAGAAATCTTGCAAGGGTTCGGTGTTGCTATAA
AGATGGGTGCC
Oktoberfest       GTTGGTGATTCCTCTGCAGAAATCTTGCAAGGGTTCGGTGTTGCTATAA
AGATGGGTGCC
Brettanomyces     GTTGGTGATTCCTCTGCAGAAATCTTGCAAGGGTTCGGTGTTGCTATAA
AGATGGAAAAT
                  **************************************************
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London            ACTAAGGCTGATTTCGATAATTGTGTTGCTATTCATCCGACTAGCGCAG
AAGAATTGGTT
California        ACTAAGGCTGATTTCGATAATTGTGTTGCTATTCATCCGACTAGCGCAG
AAGAATTGGTT
Oktoberfest       ACTAAGGCTGATTTCGATAATTGTGTTGCTATTCATCCGACTAGCGCAG
AAGAATTGGTT
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Brettanomyces      TTGGGCCCCAA-----------------------------------------
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London             ACTATGAGATGA
California         ACTATGAGATGA
Oktoberfest        ACTATGAGAAA-
Brettanomyces      ------------
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CLUSTAL 2.1 SOD1 multiple sequence alignment

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London             ------------------------------
ATGGTTCAAGCAGTCGCAGTGTTAAAGGGT
California         ------------------------------
ATGGTTCAAGCAGTCGCAGTGTTAAAGGGT
Oktoberfest        ------------------------------
ATGGTTCAAGCAGTCGCAGTGTTAAAGGGT
Brettanomyces      CCCCCCCAAATAAAACATAATTAATTTATAATGGTTCAAGCAGTCGCAG
TGTTAAAGGGT
                                                  ******************
***********

London             GATGCCGGTGTCTCTGGTGTTGTCAAGTTCGAACAGGCTTCCGAATCCG
AGCCAACCACT
California         GATGCCGGTGTCTCTGGTGTTGTCAAGTTCGAACAGGCTTCCGAATCCG
AGCCAACCACT
Oktoberfest        GATGCCGGTGTCTCTGGTGTTGTCAAGTTCGAACAGGCTTCCGAATCCG
AGCCAACCACT
Brettanomyces      GATGCCGGTGTCTCTGGTGTTGTCAAGTTCGAACAGGCTTCCGAATCCG
AGCCAACCACT
                   **************************************************
***********

London             GTCTCTTACGAGATCGCTGGTAACAGTCCTAACGCAGAACGTGGGTTCC
ACATTCATGAG
California         GTCTCTTACGAGATCGCTGGTAACAGTCCTAACGCAGAACGTGGGTTCC
ACATTCATGAG
Oktoberfest        GTCTCTTACGAGATCGCTGGTAACAGTCCTAACGCAGAACGTGGGTTCC
ACATTCATGAG
Brettanomyces      GTCTCTTACGAGATCGCTGGTAACAGTCCTAACGCAGAACGTGGGTTCC
ACATTCATGAG
                   **************************************************
***********

London             TTTGGAGATGCCACCAATGGTTGTGTCTCTGCTGGTCCTCACTTCAATC
CTTTCAAGAAG
California         TTTGGAGATGCCACCAATGGTTGTGTCTCTGCTGGTCCTCACTTCAATC
CTTTCAAGAAG
Oktoberfest        TTTGGAGATGCCACCAATGGTTGTGTCTCTGCTGGT----
ACTTCAATCCTTTCAAGAAG
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Brettanomyces      TTTGGAGATGCCACCAATGGTTGTGTCTCTGCTGGTCCTCACTTCAATC
CTTTCAAGAAG
                   ************************************    *********
**********

London             --
ACACATGGTGCTCCAACTGACGAAGTCAGACATGTCGGTGACATGGGTAACGTAAAGA
California         --
ACACATGGTGCTCCAACTGACGAAGTCAGACATGTCGGTGACATGGGTAACGTAAAGA
Oktoberfest        TTACACATGGTGCTCCAACTGACGAAGTCAGACATGTCGGTGACATGGG
TAACGTAAAGA
Brettanomyces      --
ACACATGGTGCTCCAACTGACGAAGTCAGACATGTCGGTGACATGGGTAACGTAAAGA
                     *********************************************
**********

London             CGGACGAAAATGGTGTGGCCAAGGGCTCCTTCAAGGACTCTTTGATCAA
GCTTATCGGTC
California         CGGACGAAAATGGTGTGGCCAAGGGCTCCTTCAAGGACTCTTTGATCAA
GCTTATCGGTC
Oktoberfest        CGGACGAAAATGGTGTGGCCAAGGGCTCCTTCAAGGACTCTTTGATCAA
GCTTATCGGTC
Brettanomyces      CGGACGAAAATGGTGTGGCCAAGGGCTCCTTCAAGGACTCTTTGATCAA
GCTTATCGGTC
                   ************************************************
**********

London             CTACCTCCGTTGTAGGCAGAAGCGTCGTTATCCACGCCGGCCAAGATGA
CTTAGGTAAGG
California         CTACCTCCGTTGTAGGCAGAAGCGTCGTTATCCACGCCGGCCAAGATGA
CTTAGGTAAGG
Oktoberfest        CTACCTCCGTTGTAGGCAGAAGCGTCGTTATCCACGCCGGCCAAGATGA
CTTAGGTAAGG
Brettanomyces      CTACCTCCGTTGTA--
CAGAAGCGTCGTTATCCACGCCGGCCAAGATGACTTAGGTAAGG
                   **************  *********************************
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London             GTGACACTGAAGAATCTTTGAAGACTGGTAATGCCGGTCCAAGACCAGC
CTGTGGTGTCA
California         GTGACACTGAAGAATCTTTGAAGACTGGTAATGCCGGTCCAAGACCAGC
CTGTGGTGTCA
Oktoberfest        GTGACACTGAAGAATCTTTGAAGACTGGTAATGCCGGTCCAAGACCAGC
CTGTGGTGTC-
Brettanomyces      GTGACACTGAAGAATCTTTGAAGACTGGTAATGCCGGTCCAAGACCAGC
CTGTGGTGTCA
                   ************************************************
**********

London             TTGGTCTAACCAACTAA----------------
California         TTGGTCTAACCAACTAA----------------
Oktoberfest        TTGGTCTAACCAACTAA----------------
Brettanomyces      TTGGTCTGGGGGGGAAAAATTTTTCCCGGTATA
                   *******        **
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MEGAX GSH1 Translated Protein Sequence

>California Ale GSH1
MGLLALGTPLQWFESRTYNEHIRDEGIEQLLYIFQAAGKRDNDPLFWGDELEYMVVDFDDKERNSMLD
VCHDKILTELNMEDSSLCEANDVSFHPEYGRYMLEATPASPYLNYVGSYVEVNMQKRRAIAEYKLSEY
ARQDSKNNLHVGSRSVPLTLTVFPRMGCPDFINIKDPWNHKNAASRSLFLPDEVINRHVRFPNLTASI
RTRRGEKVCMNVPMYKDIATPETDDSIYDRDWFLPEDKEAKLASKPGFIYMDSMGFGMGCSCLQVTFQ
APNINKARYLYDALVNFAPIMLAFSAAAPAFKGWLADQDVRWNVISGAVDDRTPKERGVAPLLPKYNK
NGFGGIAKDVQDKVLEIPKSRYSSVDLFLGGSKFFNRTYNDTNVPINEKVLGRLLENDKAPLDYDLAK
HFAHLYIRDPVSTFEELLNQDNKTSSNHFENIQSTNWQTLRFKPPTQQATPDKKDSPGWRVEFRPFEV
QLLDFENAAYSVLIYLIVDSILTFSDNINAYIHMSKVWENMKIAHHRDAILFEKFHWKKSFRNDTDVE
TEDYSISEIFHNPENGIFPQFVTPILCQKGFVTKDWKELKHSSKHERLYYYLKLISDRASGELPTTAK
FFRNFVLQHPDYKHDSKISKSINYDLLSTCDRLTHLDDSKGELTSFLGAEIAEYVKKNKPSIESKC*
>London Ale GSH1
MGLLALGTPLQWFESRTYNEHIRDEGIEQLLYIFQAAGKRDNDPLFWGDELEYMVVDFDDKERNSMLD
VCHDKILTELNMEDSSLCEANDVSFHPEYGRYMLEATPASPYLNYVGSYVEVNMQKRRAIAEYKLSEY
ARQDSKNNLHVGSRSVPLTLTVFPRMGCPDFINIKDPWNHKNAASRSLFLPDEVINRHVRFPNLTASI
RTRRGEKVCMNVPMYKDIATPETDDSIYDRDWFLPEDKEAKLASKPGFIYMDSMGFGMGCSCLQVTFQ
APNINKARYLYDALVNFAPIMLAFSAAAPAFKGWLADQDVRWNVISGAVDDRTPKERGVAPLLPKYNK
NGFGGIAKDVQDKVLEIPKSRYSSVDLFLGGSKFFNRTYNDTNVPINEKVLGRLLENDKAPLDYDLAK
HFAHLYIRDPVSTFEELLNQDNKTSSNHFENIQSTNWQTLRFKPPTQQATPDKKDSPGWRVEFRPFEV
QLLDFENAAYSVLIYLIVDSILTFSDNINAYIHMSKVWENMKIAHHRDAILFEKFHWKKSFRNDTDVE
TEDYSISEIFHNPENGIFPQFVTPILCQKGFVTKDWKELKHSSKHERLYYYLKLISDRASGELPTTAK
FFRNFVLQHPDYKHDSKISKSINYDLLSTCDRLTHLDDSKGELTSFLGAEIAEYVKKNKPSIESKC*

>Oktoberfest GSH1
MGLLALGTPLQWFESRTYNEHIRDEGIEQLLYIFQAAGKRDNDPLFWGDELEYMVVDFDDKERNSMLD
VCHDKILTELNMEDSSLCEANDVSFHPEYGRYMLEATPASPYLNYVGSYVEVNMQKRRAIAEYKLSEY
ARQDSKNNLHVGSRSVPLTLTVFPRMGCPDFINIKDPWNHKNAASRSLFLPDEVINRHVRFPNLTASI
RTRRGEKVCMNVPMYKDIATPETDDSIYDRDWFLPEDKEAKLASKPGFIYMDSMGFGMGCSCLQVTFQ
APNINKARYLYDALVNFAPIMLAFSAAAPAFKGWLADQDVRWNVISGAVDDRTPKERGVAPLLPKYNK
NGFGGIAKDVQDKVLEIPKSRYSSVDLFLGGSKFFNRTYNDTNVPINEKVLGRLLENDKAPLDYDLAK
HFAHLYIRDPVSTFEELLNQDNKTSSNHFENIQSTNWQTLRFKPPTQQATPDKKDSPGWRVEFRPFEV
QLLDFENAAYSVLIYLIVNSILTFSDNINAYIHMSKVWENMKIAHHRDAILFEKFHWKKSFRNDTDVE
TEDYSISEIFHNPENGIFPQFVTPILCQKGFVTKDWKELKHSSKHERLYYYLKLISDRASGELPTTAK
FFRNFVLQHPDYKHDSKISKSINYDLLSTCDRLTHLDDSKGELTSFLGAEIAEYVKKNKPSIESKC*

MEGAX GSH2 Translated Protein Sequence

>California Ale GSH2
MAHYPPSKDQLNELIQEVNQWAITNGLSMYPPKFEENPSNASVSPVTIYPTPIPRKCFDEAVQIQPVF
NELYARITQDMAQPDSYLHKTTEALALSDSEFTGKLWSLYLATLKSAQYKKQNFRLGIFRSDYLIDKK
KGTEQIKQVEFNTVSVSFAGLSEKVDRLHSYLNRANKYDPKGPIYNDQNMVISDSGYLLSKALAKAVE
SYKSQQSSSTTSDPIVAFIVQRNERNVFDQKVLELNLLEKFGTKSVRLTFDDVNDKLFIDDKTGKLFI
RDTEQEIAVVYYRTGYTTTDYTSEKDWEARLFLEKSFAIKAPDLLTQLSGSKKIQQLLTDEGVLGKYI
SDAEKKSSLLKTFVKIYPLDDTKLGREGKRLALSEPSKYVLKPQREGGGNNVYKENIPNFLKGIEERH
WDAYILMELIEPELNENNIILRDNKSYNEPIISELGIYGCVLFNDEQVLSNEFSGSLLRSKFNTSNEG
GVAAGFGCLDSIILY*

>London Ale GSH2

MAHYPPSKDQLNELIQEVNQWAITNGLSMYPPKFEENPSNASVSPVTIYPTPIPRKCFDEAVQIQPVF
NELYARITQDMAQPDSYLHKTTEALALSDSEFTGKLWSLYLATLKSAQYKKQNFRLGIFRSDYLIDKK
KGTEQIKQVEFNTVSVSFAGLSEKVDRLHSYLNRANKYDPKGPIYNDQNMVISDSGYLLSKALAKAVE
SYKSQQSSSTTSDPIVAFIVQRNERNVFDQKVLELNLLEKFGTKSVRLTFDDVNDKLFIDDKTGKLFI
RDTEQEIAVVYYRTGYTTTDYTSEKDWEARLFLEKSFAIKAPDLLTQLSGSKKIQQLLTDEGVLGKYI
SDAEKKSSLLKTFVKIYPLDDTKLGREGKRLALSEPSKYVLKPQREGGGNNVYKENIPNFLKGIEERH
WDAYILMELIEPELNENNIILRDNKSYNEPIISELGIYGCVLFNDEQVLSNEFSGSLLRSKFNTSNEG
GVAAGFGCLDSIILY*

>Oktoberfest GSH2
MAHYPPSKDQLNELIQEVNQWAITNGLSMYPPKFEENPSNASVSPVTIYPTPIPRKCFDEAVQIQPVF
NELYARITQDMAQPDSYLHKTTEALALSDSEFTGKLWSLYLATLKSAQYKKQNFRLGIFRSDYLIDKK
KGTEQIKQVEFNTVSVSFAGLSEKVDRLHSYLNRANKYDPKGPIYNDQNMVISDSGYLLSKALAKAVE
SYKSQQSSSTTSDPIVAFIVQRNERNVFDQKVLELNLLEKFGTKSVRLTFDDVNDKLFIDDKTGKLFI
RDTEQEIAVVYYRTGYTTTDYTSEKDWEARLFLEKSFAIKAPDLLTQLSGSKKIQQLLTDEGVLGKYI
SDAEKKSSLLKTFVKIYPLDDTKLGREGKRLALSEPSKYVLKPQREGGGNNVYKENIPNFLKGIEERH
WDAYILMELIEPELNENNIILRDNKSYNEPIISELGIYGCVLFNDEQVLSNEFSGSLLRSKFNTSNEG
GVAAGFGCLDSIILY*

>Brettanomyces bruxellensis GSH2
MAHYPPSKDQLNELIQEVNQWAITNGLSMYPPKFEENPSNASVSPVTIYPTPIPRKCFDEAVQIQPVF
NELYARITQDMAQPDSYLHKTTENALALSDSEFTGKLWSLYLATLKSAQYKKQNFRLGIFRSDYLIDK
KNKGTEQIKQVEFNTVSVSFAGLSEKVDRLHSYLNRANKYDPKGPIYNDQNMVISDSGYLNLSKALAK
AVESYKSQQSSSTTSDPIVAFIVQRNERNVFDQKVLELNLLEKFGTKSVRLTFDDVNDKLFIDDKTGK
LFIRDTEQEIAVVYYRTGYTTTDYTSEKDWEARLFLEKSFAIKAPDLLTQLSGSKKIQQLLTDEGVLG
KYISDAEKKSSLLKTFVKIYPLDDTKLGREGKRLALSEPSKYVLKPQREGGGNNVYKENIPNFLKGIE
ERHWDAYILMELIEPELNENNIILRDNKSYNEPIISELGIYGCVLFNDEQVLSNEFSGSLLRSKFNTS
NEGGVAAGFGCLDSIILY

MEGAX GLR1 Translated Protein Sequence

>California Ale GLR1
MLSATKQTFRSLQIRTMSTNTKHYDYLVIGGGSGGVASARRAASYGAKTLLVEAKALGGTCVNVGCVP
KKVMWYASDLATRVSHANEYGLYQNLPLDKEHLTFNWPEFKQKRDAYVHRLNGIYQKNLEKEKVDVVF
GWARFNKDGNVEVQKRDNTTEVYSANHILVATGGKAIFPENIPGFELGTDSDGFFRLEEQPKKVVVVG
AGYIGIELAGVFHGLGSETHLVIRGETVLRKFDECIQNTITDHYVKEGINVHKLSKIVKVEKNVETDK
LKIHMNDSKSIDDVDELIWTIGRKSHLGMGSENVGIKLNSHDQIIADEYQNTNVPNIYSLGDVVGKVE
LTPVAIAAGRKLSNRLFGPEKFRNDKLDYENVPSVIFSHPEAGSIGISEKEAIEKYGKENIKVYNSKF
TAMYYAMLSEKSPTRYKIVCAGPNEKVVGLHIVGDSSAEILQGFGVAIKMGATKADFDNCVAIHPTSA
EELVTMR*

>London Ale GLR1
MLSATKQTFRSLQIRTMSTNTKHYDYLVIGGGSGGVASARRAASYGAKTLLVEAKALGGTCVNVGCVP
KKVMWYASDLATRVSHANEYGLYQNLPLDKEHLTFNWPEFKQKRDAYVHRLNGIYQKNLEKEKVDVVF
GWARFNKDGNVEVQKRDNTTEVYSANHILVATGGKAIFPENIPGFELGTDSDGFFRLEEQPKKVVVVG
AGYIGIELAGVFHGLGSETHLVIRGETVLRKFDECIQNTITDHYVKEGINVHKLSKIVKVEKNVETDK
LKIHMNDSKSIDDVDELIWTIGRKSHLGMGSENVGIKLNSHDQIIADEYQNTNVPNIYSLGDVVGKVE
LTPVAIAAGRKLSNRLFGPEKFRNDKLDYENVPSVIFSHPEAGSIGISEKEAIEKYGKENIKVYNSKF
TAMYYAMLSEKSPTRYKIVCAGPNEKVVGLHIVGDSSAEILQGFGVAIKMGATKADFDNCVAIHPTSA
EELVTMR*

>Oktoberfest GLR1
MLSATKQTFRSLQIRTMSTNTKHYDYLVIGGGSGGVASARRAASYGAKTLLVEAKALGGTCVNVGCVP
KKVMWYASDLATRVSHANEYGLYQNLPLDKEHLTFNWPEFKQKRDAYVHRLNGIYQKNLEKEKVDVVF
GWARFNKDGNVEVQKRDNTTEVYSANHILVATGGKAIFPENIPGFELGTDSDGFFRLEEQPKKVVVVG
AGYIGIELAGVFHGLGSETHLVIRGETVLRKFDECIQNTITDHYVKEGINVHKLSKIVKVEKNVETDK
LKIHMNDSKSIDDVDELIWTIGRKSHLGMGSENVGIKLNSHDQIIADEYQNTNVPNIYSLGDVVGKVE
LTPVAIAAGRKLSNRLFGPEKFRNDKLDYENVPSVIFSHPEAGSIGISEKEAIEKYGKENIKVYNSKF
TAMYYAMLSEKSPTRYKIVCAGPNEKVVGLHIVGDSSAEILQGFGVAIKMGATKADFDNCVAIHPTSA
EELVTMR*

>Brettanomyces bruxellensis GLR1
MLSATKQTFRSLQIRTMSTNTKHYDYLVIGGGSGGVASARRAASYGAKTLLVEAKALGGTCVNVGCVP
KKVMWYASDLATRVSHANEYGLYQNLPLDKEHLTFNWPEFKQKRDAYNVHRLNGIYQKNLEKEKVDVV
FGWARFNKDGNVEVQKRDNTTEVYSANHILVATGGKAIFPENIPGFELGTDSDGFFRLEEQPKKVVVV
GAGYIGIELAGVFHGLGSETHLVIRGETVLRKFDECIQNTITDHYVKEGINVHKLSKIVKVEKNVETD
KLKIHMNDSKSIDDVDELIWTIGRKSHLGMGSENVNIKLNSHDQIIADEYQNTNVPNIYSLGDVVGKV
ELTPVAIAAGRKLNSNRLFGPEKFRNDKLDYENVPSVIFSHPEAGSIGISEKEAIEKYGKENIKVYNS
KFTAMYYAMLSEKSPTRYKIVCAGPNEKVVGLHIVGDSSAEILQGFGVAIKMGATKADFDNCVAIHPT
SAEELVTMR*

MEGAX SOD1 Translated Protein Sequence

>California Ale SOD1
MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPF
KKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKGDTEESL
KTGNAGPRPACGVIGLTN*

>London Ale SOD1
MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPF
KKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKGDTEESL
KTGNAGPRPACGVIGLTN*

>Oktoberfest SOD1
MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPF
KKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKGDTEESL
KTGNAGPRPACGVIGLTN*

>Brettanomyces bruxellensis SOD1
MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPF
KKTHGAPTDEVRHVDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKGDTEESLK
TGNAGPRPACGVIGLTN*

GSH Pathway Characteristics

The naming of yeast strains generally includes an alphanumeric combination that is unique to the laboratory of origin, followed by the genotype [14]. The specific three-letter combination associated with the abbreviated acronym (*e.g.*, GSH1) typically relates to gene function, protein structure, or mutant phenotype [14].

The Open Reading Frame (ORF) connotations provided are useful for highlighting a continuous stretch of codons that begins with a start codon and ends at a stop codon, indicating where translation starts. The ORF is useful for analyzing complex biological data but lacks functional information [14].

Lastly, the length of individual genes or an organism's entire genome is measured in base pairs with the total number of base pairs equal to the total number of nucleotides in one strand of DNA [14].

Gene Overview
GSH1/ YJL101C (Systematic Name)
GSH1 Location: Chromosome X 234320...236256
Open Reading Frame: Gamma glutamylcysteine synthetase
Coordinates: 234320...236256
Length: 2037 bp
GSH2/ YOL049W
GSH2 Location: Chromosome XV 238619...240094
Open Reading Frame: Glutathione synthetase
Coordinates: 238619...240094
Length: 1476 bp

GLR1/ YPL091W
GLR1 Location: Chromosome XVI 375502...376953
Open Reading Frame: Cytosolic and mitochondrial glutathione oxidoreductase
Coordinates: 375502...376953
Length: 1452 bp

SOD1/ YJR104C
SOD1 Location: Chromosome X 622550...623014
Open Reading Frame: Cytosolic copper-zinc superoxide dismutase
Length: 465 bp

DNA Matrices

The evolutionary history of these genes was inferred by using the Tamura-Nei model and the Maximum Likelihood method [33]. These evolutionary methods were conducted using the Mega X software [33].

MX: Pairwise Distances (GSH

1. California Ale GSH1 Contig				
2. London Ale GSH1 Contig	307.54			
3. Oktoberfest GSH1 Contig	629.00	828.71		
4. Heterobasidion irregulare Contig	2.38	2.38	2.38	

MX: Pairwise Distances (GSH2 Contigs13.meg)

1. California Ale GSH2 Contig					
2. London Ale GSH2 Contig	0.0000000000				
3. Oktoberfest GSH2 Contig	0.5721078609	0.5721078609			
4. Brettanomyces bruxellensis GSH2 Contig	0.5721078609	0.5721078609	0.0000000000		
5. Babesia ovata GSH2	7.9067251038	7.9067251038	7.9067251038	7.9067251038	

MX: Pairwise Distances (GLR1 Contigs 2068.meg)

1. London Ale GLR1 Contig					
2. California Ale GLR1 Contig	0.0000000000				
3. Oktoberfest GLR1 Contig	0.0407451740	0.0407451740			
4. Brettanomyces bruxellensis GLR1 Contig	0.0407451740	0.0407451740	0.0000000000		
5. Synchytrium microbalum GLR1 Contig	2.8344930099	2.8344930099	2.9622957854	2.9622957854	

MX: Pairwise Distances (SOD1 Contigs13.meg)

1. London SOD1 Contig					
2. California SOD1 Contig	0.0000000000				
3. Oktoberfest SOD1 Contig	8.0816058637	8.0816058637			
4. Brettanomyces bruxellensis SOD1 Contig	10.4875415934	10.4875415934	7.7876613373		
5. Debaryomyces hansenii SOD1 Contig	10.9114574742	10.9114574742	9.3503280274	12.1201499251	

GLOSSARY

- Allopatric speciation (*adjective*): also referred to as geographic speciation is a mode of speciation that occurs when biological populations of the same species become isolated from each other to an extent that prevents or interferes with gene flow.
- Allopolyploid (*adjective*): a strain whose chromosomes are composed of more than two genomes.
- Analogous (*adjective*): performing a similar function but having a different evolutionary origin, such as the wings of insects and birds.
- Approximate likelihood ratio test (*noun*): a statistical test used for comparing the accuracy of statistical models. The ratio expresses how many times more likely one outcome is to occur as opposed to another.
- Branch lengths (*noun*): show the path of transmission of genetic information to the next. Branch lengths indicate genetic change, the longer the branch, the more the genetic change (divergence) has occurred. The extent of genetic change is measured by estimating the average number of nucleotide substitutions per site.
- Contig (*noun*): a contig is a set of overlapping DNA segments that together represents a continuous region of DNA
- Convergent evolution (*adjective*): is the process whereby organisms not closely related, independently evolve similar traits as a result of having to adapt to similar environments or ecological niches
- Fermentation fitness (*adjective*): an organism's ability to survive and reproduce under industrial conditions.
- Gene flow (*adjective*): is the transfer of genetic variation from one population to another.

- Gene sequencing (*verb*): the process of ascertaining the sequence of nucleotides in a segment of DNA
- Homologous (*adjective*): similar in position, structure, and evolutionary origin but not necessarily in function
- Industrial Fermentation (*noun*): the intentional use of fermentation by microorganisms such as fungi and bacteria, to make products useful to humans
- Marker assisted selection (*adverb*): an indirect selection process where a trait of interest is selected based on a marker (morphological, biochemical, or DNA/RNA variation) linked to a trait of interest.
- Out-group (*adjective*): an organism outside the group of interest. All members of the group of interest are more closely related to each other than they are to the outgroup.
- Pairwise alignments (*noun*): an alignment used to identify regions of similarity that may indicate function or evolutionary relationships between two or more biological sequences.
- Primer activation (*verb*): the action of making a primer more dilute through the addition of water
- Repitched (*verb*): the process of adding yeast to wort to start fermentation and produce beer.
- Speciation (*adverb*): is the formation of a new and distinct species over the course of evolution.
- Specific gravity (*adjective*): refers to the total amount of dissolved solids or sugars in water.
- Steeped (*verb*): to soak in water

- Wort (*noun*): a sweet liquid drained from ground malt or other grains and fermented to make beer and whiskey.