

Chapter 2

***Saccharomyces cerevisiae*: Gene Annotation and Genome Variability, State of the Art Through Comparative Genomics**

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Abstract

In the early days of the yeast genome sequencing project, gene annotation was in its infancy and suffered the problem of many false positive annotations as well as missed genes. The lack of other sequences for comparison also prevented the annotation of conserved, functional sequences that were not coding. We are now in an era of comparative genomics where many closely related as well as more distantly related genomes are available for direct sequence and synteny comparisons allowing for more probable predictions of genes and other functional sequences due to conservation. We also have a plethora of functional genomics data which helps inform gene annotation for previously uncharacterised open reading frames (ORFs)/genes. For *Saccharomyces cerevisiae* this has resulted in a continuous updating of the gene and functional sequence annotations in the reference genome helping it retain its position as the best characterized eukaryotic organism's genome. A single reference genome for a species does not accurately describe the species and this is quite clear in the case of *S. cerevisiae* where the reference strain is not ideal for brewing or baking due to missing genes. Recent surveys of numerous isolates, from a variety of sources, using a variety of technologies have revealed a great deal of variation amongst isolates with genome sequence surveys providing information on novel genes, undetectable by other means. We now have a better understanding of the extant variation in *S. cerevisiae* as a species as well as some idea of how much we are missing from this understanding. As with gene annotation, comparative genomics enhances the discovery and description of genome variation and is providing us with the tools for understanding genome evolution, adaptation and selection, and underlying genetics of complex traits.

Key words: Gene annotation, comparative genomics, next generation sequencing, novel genes, genetic variation.

1. Introduction

Gene annotation and genome variation are interrelated and each can inform the other (*see Fig. 2.1*). To address the question of “When is a gene not a gene?” for dealing with dubious

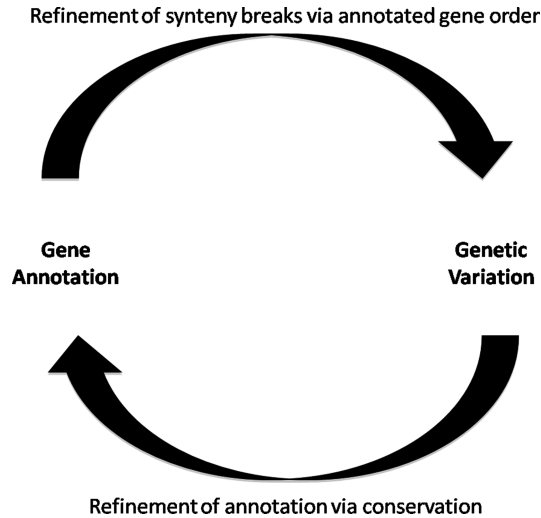


Fig. 2.1. Gene annotation and genetic variation inform each other. It has become clear that the increased number of genome sequences from different strains and species of *Saccharomyces* has been very informative to gene annotation through comparative genomics. This has led to a continuous evolution of the annotation of the reference *S. cerevisiae* genome that has in turn led to the annotations of related genomes. Less well appreciated is the role annotated genomes have played in our understanding of genetic variation, in particular structural variation, due to insertions and deletions as well as rearrangements such as translocations. Annotated genomes allow for quick and efficient alignments, such as through the Yeast Gene Order Browser (*see text*), without the need for multi-sequence alignments. Potential areas of interest are immediately obvious as apparent synteny breakpoints or as duplicated regions.

open reading frames, comparative studies with several strains and species can be a powerful tool, obviating the need for experimental determination of function. Similarly, gene annotations embedded in a gene order or synteny browser can be used to determine areas of genome variation, in terms of insertions, deletions, and rearrangements. Finally, for uncharacterised yet conserved genes, the cross referencing of the vast amount of functional genomic data can inform and improve functional annotation.

2. Gene Annotation

The reference genome, S288C, is constantly changing with updates in both sequences and annotations (1). Many of the updated gene annotations come from functional studies but others come from informatic analysis of genome comparisons with functional follow-ups. These updates can be seen for each annotated gene or sequence element at SGD (*see* <http://www.yeastgenome.org/cgi-bin/seqTools>) and a global

picture of the updates can be found at <http://www.yeastgenome.org/cache/genomeSnapshot.html>. The rate of change in annotations is not really slowing down as there are still many functions to be determined and many open reading frames still annotated as dubious, as there is no corroborative evidence for them actually being genes. New and improved annotation pipelines are in place to improve functional annotations as more data becomes available (2, 3).

Genome comparisons of related species have given the greatest advance in gene annotation in recent years (4, 5), and numerous ORFs are less likely to be real based on lack of conservation (Fig. 2.1). The reduction of the original 6,200 ORFs by 15% is in large part due to comparative genomics. On the flip side, small ORFs, which did not pass the threshold for being annotated as possible ORFs, are now properly annotated due to conservation in related species (1), which is thought to be due to functional constraints. This sequence conservation has also led to the annotation of non-coding sequence elements, either presumed functional elements or discovery of new elements including transcription factor binding sites (4, 6, 7).

2.1. Gene Annotation Informing Genome Variation

Annotated genomes have been useful in comparative genomics, particularly in determining ancestral structural states and the sequence of gross chromosomal rearrangement events that lead to the extant genome of *Saccharomyces cerevisiae* as well as its relatives. The determination of the Whole Genome Duplication by Wolfe and Shields (8) is a case in point, where genome dynamics and variation over evolutionary time have been determined using comparisons of annotated regions which have since developed into an annotated gene order browser (9, 10). Since then the use of a gene order browser has become a standard tool for analysis of genome variation between species.

Sometimes the use of such tools can lead to apparent inconsistencies between studies. When comparing the genomes of the *Saccharomyces sensu stricto* species, it is clear that most of the genome is syntenic and that there are only a few gross chromosomal rearrangements. Mapping the breakpoints by physical means can narrow down the sites of ancestral rearrangements within a few kilo-base pairs. In the case of *Saccharomyces bayanus*, eight breakpoints for four reciprocal translocations were determined (11). When the same isolate was sequenced at low coverage and the reads annotated where ORFs were found, a comparison to the gene order browser for *S. cerevisiae* resulted in the determination of at least 45 breakpoints as ORFs were found in the same clone whose homologues in *S. cerevisiae* were located far apart, mostly on other chromosomes (12). A closer inspection revealed that there was a lot of differential accumulation of divergence in one or the other of a pair of gene duplicates after the

whole genome duplication resulting in gene relics that were not annotated (13). What looked like gross chromosomal rearrangements in many cases was loss of open reading frames differentially between duplicated segments in the two species and a sequence comparison revealed that synteny was retained.

Gene annotation has improved but is still an evolving process. Various projects for improving automated gene annotation are underway at many places and these rely in a large part on comparative genomics. It is likely that proper annotation will continue to require human intervention. The state of the art for functional genome annotation in *S. cerevisiae* utilizes high-throughput experimental data as well as computational predictions (2, 3).

2.2. Gene Annotation Challenges

Some genome regions are particularly problematic, as they do not assemble well, making annotations difficult if not impossible. In particular, the subtelomeric regions generally are not included in genome projects due to technical difficulties in cloning, sequencing, and eventual assembly. For *S. cerevisiae* this is of particular importance as many of the genes responsible for important phenotypic variation, such as brewing, baking and general fermentation properties, are located in the subtelomeres. The reference genome for *S. cerevisiae* remains the only finished genome as its subtelomeres were individually marked, cloned, and sequenced during the genome project, an effort that cannot be done efficiently for any other genomes. None of the other yeast strains (14–16) (*see also* http://www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae.3/Info.html) and species (4, 6, 7) sequenced have assembled and therefore annotated subtelomeres making it difficult to progress with many important studies.

3. Genome Variation

Genome variation has always been underlying studies in *S. cerevisiae* but has not always been taken into account. For example, studies using the reference genome as a template for experiments on another strain may not result in interpretable data for regions of the genomes that are different. In particular the subtelomeric regions as mentioned above vary a great deal between strains and therefore it is not possible to determine which chromosome end or which copy of a gene is responsible for the data. This is illustrated in studies of meiotic double strand breaks, which are generally done in strain SK1, which varies greatly in its subtelomeres from the reference genome (14, 17). The data from array-based analyses therefore cannot be interpreted in these regions (18).

Table 2.1
Techniques for assessing genetic variation

Technique	Uses	Disadvantages
Pulsed field gel analysis	Gross chromosomal rearrangements and chromosome length polymorphisms	Low resolution, not high throughput
AFLPs	Phylo-geographic relationships of closely related strains/species	Not good for distantly related species due to loss of homology with phylogenetic distance
Microsatellites	High-throughput assessment of relatedness of strains using multiple alleles at few loci	Low resolution in terms of genome coverage, identical alleles not necessarily identical by descent
Microarrays – ORFs/long oligos	High throughput, copy number variation (CNV), presence/absence of sequences	Cannot assess unknown sequence, not good for SNP variation
High-density microarrays	High throughput, CNV, presence/absence as well as determination of sequence variants (SNPs)	Cannot assess unknown sequence
Whole genome sequencing	Highest resolution, can assess novel previously unknown sequence	Expensive, throughput depends on ability to multiplex

We now have the capability of assessing variation amongst *S. cerevisiae* strains and related species by a variety of means. Currently in use are physical analysis of structural variation using pulsed field gels and Southern analysis (11, 17, 19–22), amplified fragment length polymorphisms (AFLPs) (23–25), microsatellite variation at several sites across the genome using PCR (26–28), presence/absence as well as SNP detection using microarrays (29–35) and finally sequencing at either several loci (36–39) or the whole genome (14) (*see Table 2.1*).

3.1. Genome Variation by Pulsed Field Gel Electrophoresis

The use of pulsed field gel electrophoresis to separate large DNA molecules has proven very useful in looking at structural variation in yeast genomes: assessing variation amongst isolates (17, 19, 21, 22), variation generated by genome instability (20), or variation generated over evolutionary time (11, 17). The resolution of pulsed field gels coupled with Southern analysis is quite low; however, a great deal of effort is required to narrow down breakpoints (11). It is also difficult to scale up to large numbers of samples though 10 s to 100 s are possible (40).

3.2. Genome Variation via AFLPs

A higher throughput method in use is amplified fragment length polymorphisms which is a quick method of assessing relatedness

amongst strain isolates (24, 25). This technique is invariably used along with rDNA typing or other genetic characterization to generate a more informed and consistent picture of relationships. One of the problems with the technique is it is difficult to know what is actually being compared as these are randomly amplified fragments, and how much of the genome is being assessed. Another difficulty is that the method is limited to close relatives as increased phylogenetic distances reduce the likelihood that fragments are comparable through homology (23).

3.3. Genome Variation via Microsatellites

One of the most efficient ways of determining general strain variation is by microsatellite analysis. The increased number of alleles available at these loci in part makes up for the lack of number of loci assessed. With only a few markers, large numbers of strains can be genotyped (26–28). Phylogenetic relationships can be inferred and some feel for diversity in the species can be obtained. There are limitations to this approach. One is that only a limited part of the genome is genotyped and in many studies not even every chromosome is marked. This severely limits the use of the genotype data for mapping genetic differences responsible for various phenotypes for example. Another is that the allele state at a microsatellite is not necessarily a good indicator of identity by descent and therefore inferred phylogenetic relationships are compromised. A particular copy number allele could have been arrived at from different “mutational” changes from different alleles. Despite these problems, microsatellite genotyping remains a quick and inexpensive way to assess diversity for large numbers of strains.

3.4. Genome Variation via Microarray Comparative Genome hybridization

A better approach but less high throughput is the use of microarrays and comparative genome hybridization. For large probes on arrays, the resolution can yield information on presence or absence as well as copy number (30). This has generally worked well for some studies but has its limitations. The main limitation is that you cannot look for sequences that are not known. These larger probe arrays are also not very useful for detecting sequence divergence. This approach is particularly suited for genome stability and/or composition studies with hybrids or conditions resulting in aneuploidies where copy number changes are important determinants. Unfortunately the microarrays cannot provide information on location or structure accompanying copy number changes. Complementary analysis with specific probes on pulsed field gels can resolve location and structural differences.

Higher resolution can be obtained with high-density arrays using short oligos that cover the whole known genome (32, 34, 35). With appropriate analysis these can even detect single SNP differences making them almost as good as sequencing (29, 34, 35). Such arrays are more expensive and therefore may

be prohibitive for large-scale studies but they provide a genome-wide assessment of variation for SNPs, small- and large-scale deletions as well as copy number to a limited extent. There is still the problem of assaying only previously known sequences.

3.5. Genome Variation via Whole Genome Sequencing (WGS)

By far the most effective assessment of genome variation is whole genome sequencing. Here the issue is balancing the cost with the value of completeness. Only sequencing will reveal novel genes and sequences that were previously unknown. Several individual *S. cerevisiae* strains have been sequenced to near completeness using first generation Sanger sequencing since the reference genome was completed (15, 16) and http://www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae.3/Info.html. These have each provided insights into genome variation such as novel genes, introgressions from outside the species as well as frequencies and types of variation. Each of these was time consuming and costly and each suffers from incompleteness of the subtelomeres as well as the large tandem arrayed sequences such as the rDNAs. Although a great deal of information can be gleaned from these sequences such as some assessment of population structure, selection and adaptation, and human influence, the limited number of sequences does not represent the species as a whole nor can it really describe population structures which require many individuals within and between to accurately determine.

One way to increase numbers of individuals without the cost of whole genomes is to sequence a few genes from various locations in the genome. This has been used effectively to describe population structures in *Saccharomyces* yeasts (36, 37, 39). This type of analysis resolves some of the issues of population structure but in many cases new questions arise.

Another approach to increase the numbers of individuals without the cost of complete genomes is low level whole genome shotgun sequence coverage that has proven very effective at determining population structure and resolving many issues about selection and human influence (14). With the assumption that there are populations of related individuals, it is possible to determine global genome-wide phylogenetic relationships without having the complete genomes. Although the difficult regions of the genomes are still not resolved, some inferences concerning regions such as subtelomeres and large tandem arrays can be made. By surveying many strains at lower coverage, the discovery of novel genes may be more efficient than complete sequences of fewer strains as populations of related individuals will share these novel genes. The combined sequence coverage within a population reduces the chances of missing novel genes. Using this approach, all six known gene families not in the reference genome were found amongst 35 other *S. cerevisiae* strains as

were the novel genes discovered in the individual near complete genomes recently sequenced. In addition 38 new genes/gene families were discovered (14). Despite most of these being in subtelomeric regions that were not assembled, the general composition of novel gene families, i.e. presence and distribution amongst strains/populations could be made. Further complementary analysis with specific probes and pulsed field gels will help with the structural analysis of these novel genes. Annotation of these genes will have the same problems as any novel potential genes and in this case comparative genomics would not be helpful, as the regions are not assembled.

In addition to novel gene discovery, population genomic sequence surveys provide evidence for sequence variation previously unknown. In the case of the rDNA array, it is generally assumed that every copy within an array has the same sequence though different strains can have different variant arrays. The population genomic survey of several strains revealed that in addition to the SNPs that varied between arrays in different strains, there were sequence differences between rDNA copies within arrays with significant frequencies (41). Despite the low coverage overall for the genomes in this survey, the coverage of rDNAs was substantial due to their large copy number. This allowed the determination of sequence variants with high accuracy both between and within arrays. What is not possible from this analysis is the determination of order of variants within an array.

The current state of the art for genome variation determination still includes microsatellite analysis (26–28), as well as microarrays (32, 34, 35), where genotyping by arrays is becoming quite sophisticated (29). The best determination, however, is still whole genome sequencing without which much information on variation is missing. Our current understanding of the population structure of *S. cerevisiae*, with several well-delineated populations and a large number of mosaic strains resulting from interbreeding between these populations (14), could not have been determined without population genomic sequencing.

4. Conclusions

Challenges and future prospects include the assembly and annotation of complex regions of the genome. This includes repetitive regions such as the rDNA arrays, for which we have seen some progress as described above, as well as subtelomeric regions, which we have seen contain many of the genes and gene families responsible for adaptive and phenotypic differences, yet are not well characterized. Second generation sequencing with increased

depth of coverage in short timeframes will be a major part of the solution, yet brings with it the additional challenges of quantity and quality of the sequence reads as well as the length of reads available. These challenges in both gene annotation and genome variation will only be met by combined approaches utilizing new sequencing technologies as well as new informatic tools for assembly, comparison, and compilation/cross referencing of diverse data sets.

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