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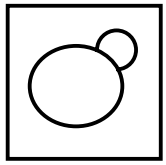

Research Article

A Re-annotation of the *Saccharomyces cerevisiae* Genome

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Abstract

Discrepancies in gene and orphan number indicated by previous analyses suggest that *S. cerevisiae* would benefit from a consistent re-annotation. In this analysis three new genes are identified and 46 alterations to gene coordinates are described. 370 ORFs are defined as totally spurious ORFs which should be disregarded. At least a further 193 genes could be described as very hypothetical, based on a number of criteria.
It was found that disparate genes with sequence overlaps over ten amino acids (especially at the N-terminus) are rare in both *S. cerevisiae* and *Sz. pombe*. A new *S. cerevisiae* gene number estimate with an upper limit of 5804 is proposed, but after the removal of very hypothetical genes and pseudogenes this is reduced to 5570. Although this is likely to be closer to the true upper limit, it is still predicted to be an overestimate of gene number. A complete list of revised gene coordinates is available from the Sanger Centre (*S. cerevisiae* reannotation: ftp://ftp/pub/yeast/SCreannotation). Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: annotation; *Schizosaccharomyces pombe*; *Sacccharomyces cerevisiae*; comparative genomics; sequence orphans; hypothetical proteins

Introduction

Background

The publication in 1996 of the first complete eukaryotic genome sequence, that of *Saccharomyces cerevisiae*, heralded a new era in biology (Goffeau *et al*., 1996). This resource not only benefited those investigating *S. cerevisiae*, but also enabled inferences from the functional data to be transferred to a diverse range of other organisms. Unexpectedly, a significant proportion (56%) of annotated genes had not been studied previously, despite more than 50 years of traditional biochemistry and genetics (Oliver *et al*., 1992, Oliver, 1996, Mewes *et al*., 1997). This observation stimulated the application of functional genomics technologies to characterise these genes and their products, either gene-by-gene in small laboratories, or on a larger scale in some research institutes (Hieter and Boguski, 1997).

In the five years since the *S. cerevisiae* genome was sequenced, the majority (70%) of the predicted genes have been assigned an initial functional characterisation in the Yeast Protein Database (YPD, Proteome Inc. **http://www.proteome.com/databases/index.html**). Establishing the functional inter-relationships between all the genes in a genome requires, in the first instance, the assignment of genes to preliminary functional classes. These initial assignments will authenticate predicted genes as coding entities and partition the data into categories for subsequent biological analyses. However, it will be difficult to assess when this milestone has been reached since the exact number of genes in *S. cerevisiae* is still unclear: the Munich Information Center for Protein Sequences (MIPS) database has a protein complement of 6368 (**http://www.mips.biochem.mpg.de/proj/yeast**) Saccharomyces Genome Database (SGD) has 6310 (**http://genome-www.stanford.edu/Saccharomyces/**), and YPD has 6142 as of 26 January 2001.

It is likely that a significant cause of the discrepancies between these gene numbers are due to small, fortuitously occurring ORFs (open reading frames), which are notoriously difficult to

distinguish from real genes (Dujon, 1996). In the original *S. cerevisiae* annotation, only ORFs greater than 100 amino acids in size were considered. This threshold was imposed in order to reduce the chance of missing small proteins without over-prediction due to the statistically expected frequency of small ORFs (Sharp and Cowe, 1991). Those without assigned function or homologues were designated *sequence orphans* (Dujon, 1996). As genome sequencing proceeded, the ratio of orphans to ORFs with homologues increased rapidly- so much so that this phenomenon was termed '*The mystery of orphans*' (Dujon, 1996).

The existence of relatively high numbers of orphans can only be attributed to one or a combination of the following:

1. They may simply be spurious ORFs. In *S. cerevisiae*, a number of predicted genes are also completely or substantially overlapping with defined coding features and should therefore be *disregarded*.
2. They may arise due to the acquisition of novel species-specific functions.
3. They escape functional characterisation by homology because they are rapidly evolving.
4. Identifiable homologues in other organisms exist, but these have not yet been sequenced.

The question of *S. cerevisiae* gene number has been addressed many times, with differing outcomes. Mackiewicz *et al.* (1999) estimated the total number of protein coding ORFs to be 4800, based on their sequence properties. Zhang and Wang (2000) calculated the likely number to be ≤ 5645, based on the assumption that unknown genes have similar statistical properties to known genes. As part of the Genolevures project, Blandin *et al.* (2000) performed a consistent re-annotation of the *S. cerevisiae* genome using uniform criteria, revealing 50 possible novel genes and 26 gene extensions. They proposed a protein coding gene set of *at least* 5600 genes. As part of the same initiative, Malpertuy *et al.* (2000) estimate that the *S. cerevisiae* genome contains 5651 actual protein coding genes (including the 50 new predictions), and that the public databases contain 612 predicted ORFs that are not protein coding.

The availability of an additional yeast genome, that of *Schizosaccharomyces pombe* (fission yeast), which has 99.5% of its coding sequence annotated and deposited in the EMBL database (manuscript in preparation), will allow the comparison of the complete genomes and proteomes of two well-studied unicellular eukaryotes, which diverged around 330 million years ago (Berbee and Taylor, 1993).

Aims

The discrepancies in gene and orphan numbers proposed by previous analyses suggested that *S. cerevisiae* would benefit from a consistent reannotation, applying new analytical methods and incorporating the data which have become available over the last four years. In doing so, we wished to achieve:

1. The refinement of gene complement.
2. The classification of orphans into hypothetical, very hypothetical, and spurious ORFs which should be disregarded.
3. The identification of gene prediction errors.
4. The identification of new genes.

The *Sz. pombe* genome annotation effort has benefited immensely from the availability of the complete genome of *S. cerevisiae*. The analysis methods used for the *Sz. pombe* genome combine *ab initio* gene prediction algorithms and homology search results with rigorous manual inspection of biological context (Xiang *et al.*, 2000). In addition, consistency checks using available cDNAs and ESTs have been routinely performed, and new experimental data from the fission yeast community immediately incorporated into the dataset. We believe that these methods provide an accurate, detailed gene set for this organism. The *Sz. pombe* analysis procedure has been applied to *S. cerevisiae* in order to define an up-to-date non-redundant gene set with consistent annotations and a new estimate of orphan numbers.

Methods

DNA sequences

The sequences of the 16 *S. cerevisiae* chromosomes, and associated ORF translations, were downloaded from SGD on the 16th November 2000. ORF coordinates were then converted into EMBL feature table format and imported into the Artemis sequence analysis and annotation tool (Rutherford *et al.*, 2000).

Analysis procedure

A number of standard analysis tools were used to assist the interpretation of the sequence data (as applied to the *Sz. pombe* genome) (Xiang *et al*., 2000). Searches were performed against public databases (SWISS-PROT and TrEMBL (Bairoch and Apweiler, 1999), EMBL (Stoesser *et al*., 1999), Pfam (Bateman *et al*., 1999), and PROSITE (Bairoch, 1994)) using standard software (BLAST (Altschul *et al*., 1990), MSPcrunch (Sonnhammer and Durbin, 1994), tRNAscan (Lowe and Eddy, 1997), FASTA (Pearson and Lipman, 1988) and Genewise (Birney *et al*., 1996)), to complete a series of automated analyses. This enabled annotated DNA and protein features to be confirmed. Other elements not included in the SGD annotation (experimentally identified snoRNAs and other cellular RNAs, omitted LTRs, and protein domains), were also mapped onto the sequence using in-house Perl scripts. *De novo* gene predictions were not performed as part of this analysis.

New genes

In the *Sz. pombe* genome, more than 300 genes have been identified which are conserved at the protein level in other organisms, but absent from the *S. cerevisiae* dataset (manuscript in preparation). Some of these were small genes (70–150 amino acids); TBLASTN searches were conducted to determine whether these small genes had been omitted from the initial *S. cerevisiae* gene predictions.

New gene coordinates

Within the annotation tool Artemis (Rutherford *et al*., 2000), FASTA alignments were performed on existing gene predictions, to assess their accuracy. Overlapping ORFs were subject to systematic manual inspection to determine whether the correction of frameshifts or sequencing errors could extend homology, by merging existing genes or increasing their length.

Disregarded spurious ORFs, overlapping with real genes

ORFs which have all, or the majority of their translation overlapping with other annotated features, were individually assessed for similarity to all organisms, as described in *New gene coordinates* above, together with experimental data if available. For ORFs to be considered as spurious, they had to meet all of the following criteria:

1. Small size (35–250 amino acids).
2. Absence of similarity to known proteins.
3. Absence of functional data which could not have been generated by the real overlapping gene.
4. Greater than 25% overlap at the N-terminus or 50% overlap at the C terminus with another coding feature; overlap with another feature at both ends; or ORF containing a tRNA.

Transposon fragments were also removed.

Very hypothetical ORFs

In *Sz. pombe*, 177 ORFs which are considered unlikely to be coding but cannot yet be dismissed as spurious have been assigned as *very hypothetical* according to the following criteria:

1. Small size (100–250 amino acids).
2. Absence of similarity to other known proteins.
3. Overlap with other features, particularly at the N-terminus, where they might interfere with promoters (the overlaps in these cases are smaller than those observed in disregarded ORFs).
4. Extreme GC content.

The annotation of *Sz. pombe* adequately discriminates between *very hypothetical proteins* and real genes and this approach has been applied to a re-annotation of the *S. cerevisiae* genome.

Results

New genes identified

Three new genes were identified; 1. *YBL071W-a* a hypothetical conserved protein (simultaneously identified by Blandin *et al*.) 2. *YAL044W-a*, the homologue of *Sz. pombe uvi31* 3. *YDL085C-a*, the homologue of the human *4F5S* disease-associated gene. The new genes and coordinates are listed in Table 1.

New coordinates (merged or extended genes)

The complete list of 46 proposed alterations to gene coordinates are presented in Table 1. Some of these changes have already been confirmed experimentally and deposited in the SWISS-PROT database

Table 1. New, merged or extended genes

Chr.	CDS	Comment	NewCoordinates
I	YAL044W-a	New gene	57520..57852
II	YBL071W-a	New gene	89973..90221
IV	YDL085C-a	New gene	302463..302669
I	YAL065C + YAL064C-A	1	11566..13173,13177..13362,13367..13744
I	YAR066W + YAR068W	1	221035..221643,221647..222930
I	IMD1	1or2	227728..228844,228846..229303
II	VMA2	extended	491228..492781
III	YCR099C + YCR100C + YCR101C	1	300825..301292,301294..302463,302478..303023
III	YCL001W-A	1or2	113074..113532,113549..113641,113644..114018
III	YCL069W	1or2	9427..9459,9463..11082
IV	PRM7 + YDL038C + YDL037C	1or2	81982..382327,382330..384596,384600..385583
IV	YDR134C	1	721064..721474
IV	YDR474C + YDR475C	1or2	1407453..1409475,1409475..1409661,1409661..1410081
IV	TTR1	trimmed	1471122..1471451
V	YER066W	1or2	289637..290797
V	HVG1 + YER039C-A	1or2	228455..229480
V	YEL077C + YEL076C + YEL075C	1	264..4095,4097..4553,4553..5117,5134..5418,5420..5875
V	YER189W + YRF1-2	1	571150..571463,571465..576520
V	KHS1	1	565667..565792,565796..566398
VI	AAD6 + YFL057C	1or2	14305..14919,14919..15431
VI	BLM3 + YFL006W	1or2	123474..128885,128885..129904
VI	YFL064C + 65C + 66C	1	1..1516,1437..3008,3033..3338,3340..3846
VI	YFR012W	1or2	167880..168488,168487..169113,169116..169301
VI	YFL042C + YFL043C	1or2	45720..46157,46156..47745
VIII	YHR218W + YHR219W	1or2	557819..557854,557857..558606,558712..559820,559822..560167,560169..562043
VIII	YHL049C + YHL050C	1or2	445..2283,2285..3700,3728..4540
VIII	YHR214W	1or2	541503..542255,542259..543542
IX	SDL1 + YIL167W	1or2	29032..29412,29416..30048
IX	NIT1 + YIL165C	1or2	33718..34086,34090..34686
IX	YIR043C + YIR044C	1or2	437040..437990,437994..438176
IX	YIL177C	1	483..4988,4987..6147
IX	HXT12	2	19515..19805,19808..21220
X	PRM10 + YJL107C	1or2	217402..218554,218554..219713
XI	PKT1 + YKT9	1or2	68270..69079,69081..70220
XI	YKL002W	new splice	437416..437475,437544..438182
XI	YKL033W-A	1or2	74144..374305,374308..374853
XII	AQY2 + YLL053C	1or2	35502..36044,36043..36360
XII	YRF1-4 + YLR462W + YLR464W	1	1065951..1066556,1066567..1067079,1067082..1071230
XII	SDC25 + YLL017W	2	112233..112502,112504..115992
XIII	YMR084W + YMR085W	1or2	436627..437412,437415..438788
XV	YOL162W + YOL163W	1or2	9596..10102,10106..10765
XV	VPS5	extended	453769..453801,453804..455795
XV	YOL048C	new splice	240202..240945,241024..241308
XV	ABP140	2	784855..785685,785687..786742
XVI	YPL275W + YPL276W	1or2	17948..18382,18386..18416,18418..19079
XVI	YPL277C + YPL278C	1or2	15053..15492,15494..16868

1 Pseudogene
2 Possible sequencing error (frameshift or stopcodon) or real mutation

(Bairoch and Apweiler, 1999) but may correspond to mutations in the sequenced strain. However, fragments pertaining to the same sequence should be represented as a single feature in the public databases. In addition to increased homology, data from YPD indicates identical phenotypes and expression patterns for some of these proposed merges. For example, *PRM7 + YDL038 + YDL037* have the same transcript profile (repressed by methylmethanesulphonate). Some of these proposed

gene extensions make previously predicted ORFs spurious.

Disregarded (spurious) ORFs

Using the criteria described in Methods, 370 ORFs were disregarded (Table 2. and see **http://www.sanger.ac.uk/Projects/S_cerevisiae/spurious.shtml**). In agreement with Blandin *et al.* (2000), the ORFs which correspond to SAGE tags within LTRs have been reclassified as spurious.

Orphans—very hypothetical

The discrimination between *S. cerevisiae* very hypothetical proteins and orphans which are more likely to be coding suggests 193 *S. cerevisiae* CDS should be described as *very hypothetical* ORFs (after the removal of ORFs which should be disregarded). Of these, 72 exhibit an overlap with another CDS (Table 3 and see **http://www.sanger.ac.uk/Projects/S_cerevisiae/veryhypothet.shtml**).

The G+C content, range and average was calculated for the fully partitioned ORFs on chromosomes I–V. ORFs were partitioned as: Real (characterised or well-conserved) = R; Sequence Orphans (possibly coding) = O; and Very Hypothetical (unlikely coding) = V. The mean G+C content for the partitioned ORF sets R : O:V are 40.24 : 40.37 : 38.84 respectively, which indicates there may be compositional differences between them. Even though the range of G+C for the very hypothetical proteins is smaller than for real (23.29 *vs.* 27.07), the sample standard deviation is greater (V = 5.06; R = 3.47).

Discussion

Novel genes

The three novel genes predicted by this analysis have now been incorporated into the MIPS database (M. Muensterkoetter, MIPS, pers comm).

Blandin *et al.* predicted 49 additional novel genes using interspecies sequence conservation, but some of these proposed new genes are spurious and others could be labelled *very hypothetical* using the criteria outlined in Methods. Some of these are predicted due to other non-CDS features. Others are extensions to existing genes. For example, *YMR013wa* is overlapped completely by a cellular RNA, *YGL258w* is part of *VPS5*, and *YER039ca* is part of *HGV1*. Other gene predictions from this dataset extend beyond the newly proposed coding region, and may correspond to regulatory regions, or to as yet undiscovered cellular RNAs. For example, *YDL159wa* is predicted to code for a 43 amino acid peptide (129 base pairs corresponding to the largest ORF) but the region of high similarity extends over 391 base pairs. Some predictions are derived from translations between 28 and 99 amino acids in length, and correspond to low complexity DNA sequence, often with only one species homologue. There are attendant risks in defining a CDS solely from an ORF and a statistically significant BLAST score (particularly with closely related organisms), as this may not always be biologically significant, or may pertain to a non-CDS feature. These predicted ORFs have been added to the Sanger annotation as miscellaneous features and will require further analysis before inclusion in the protein set.

Merged and extended genes

Of the 46 alterations we propose, eight belong to subtelomeric duplicated elements and are possibly pseudogenes. For the remainder, those sequences not already corrected or confirmed or corrected by the sequencing of the genomic DNA will require resequencing for verification. However, frameshifts may still persist in the sequenced strain due to mutations.

Disregarded spurious ORFs

Of the 370 genes proposed here to be *disregarded* ORFs, 227 were also predicted as unlikely to be coding by Zhang and Wang (2000). However, the Zhang and Wang analysis did not adequately differentiate between coding and non-coding sequences when applied to ORFs which were not in the *questionable* category of the MIPS database. Here, 18 of the 46 ORFs predicted to be non-coding for chromosomes I and II are now either functionally characterised (YPD) or conserved in distantly related organisms.

Malpertuy *et al.* (2000) propose that 91 of the ORFs annotated by MIPS as *questionable* (because they largely overlap other features) are actually real, based on similarity to the recently sequenced hemiascomycetes. We propose all of these ORFs should be *disregarded* as they will generate apparently

Table 2. Spurious ORFs within other CDS or sequence features

Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature
YAL004W	SSA1	YDL034W	GPR1	YER119C-A	SCS2
YAL035C-A	MTW1	YDL041W	SIR2	YER138W-A	LTR
YAL043C-A	ERV46	YDL050C	LHP1	YER181C	LTR
YAL045C	new UVI31	YDL062W	YDL063C	YFL013W-A	YFR055W
YAL058C-A	CNE2	YDL068W	CBS1	YFL067W	YFL013C
YAL064W-B	YAL065C	YDL094C	PMT5	YFL068W	YFL066C
YAR009C	ty fragment	YDL096C	PMT1	YFR024C	YFL066C
YAR010C	ty fragment	YDL118W	YDL119C	YFR056C	YFR024C-A
YBL012C	SCT1	YDL151C	RPC53	YGL024W	PGD1
YBL053W	SAS3	YDL152W	SAS10	YGL042C	DST1
YBL062W	SKT5	YDL158C	STE7	YGL069C	YGL068W
YBL065W	SEF1	YDL163W	CDC9	YGL072C	HSF1
YBL070C	AST1	YDL187C	YDL186W	YGL074C	HSF1
YBL073W	AAR2	YDL221W	CDC13	YGL102C	RPL28
YBL077W	ILS1	YDL228C	SSB1	YGL109W	YGL108C
YBL083C	RHK1	YDR008C	TRP1	YGL132W	YGL131C
YBL094C	YBL095W	YDR034C-A	LTR	YGL152C	CUP2 PEX14
YBL096C	YBL095W	YDR048C	YDR049W	YGL165C	CUP2
YBL100C	ATP1	YDR053W	DBF4	YGL168W	PMR1
YBL107W-A	LTR	YDR094W	YDR093W	YGL199C	YGL198W
YBR051W	REG2	YDR112W	YDR111C	YGL214W	SKI8
YBR064W	YBR063C	YDR133C	YDR134C	YGL217C	KIP3
YBR089W	POL30	YDR149C	NUM1	YGL218W	YGL219c
YBR090C	NHP6B	YDR154C	CHP1	YGL235W	MTO1
YBR099C	MMS4	YDR187C	CCT6	YGL239C	CSE1
YBR113W	CYC8	YDR193W	NUP42	YGR011W	YGR010W
YBR116C	TKL2	YDR199W	YDR200C	YGR018C	YGR017W
YBR124W	TFC1	YDR203W	RAV2	YGR022C	MLT1
YBR174C	YBR175W	YDR230W	COX20	YGR064W	SPT4
YBR206W	KTR3	YDR241W	LTR (29.16GC)	YGR073C	SMD1
YBR219C	YBR220C	YDR269C	CCC2	YGR114C	SPT6
YBR224W	YBR223C	YDR271C	CCC2	YGR115C	SPT6
YBR226C	YBR225W	YDR278C	TRNA	YGR122C-A	LTR
YBR232C	PBP2	YDR290W	RTT103	YGR137W	YGR136W
YBR266C	YBR267W	YDR327W	SKP1	YGR151C	RSR1
YBR277C	DPB3	YDR340W	TRNA and LTR	YGR160W	NSR1
YCL022C	KCC4	YDR355C	NUF1	YGR164W	TRNA
YCL023C	KCC4	YDR360W	VID21	YGR176W	ATF2
YCL041C	PDI1	YDR366C	LTR	YGR190C	HIP1
YCL042W	GLK1	YDR396W	NCB2	YGR219W	MRPI9
YCL046W	YCL045C	YDR401W	DIT2	YGR226C	AMA1
YCL074W	ty fragment	YDR413C	YDR412W	YGR228W	SMI1
YCL075W	ty fragment	YDR417C	RPL12B	YGR242W	YAP1802
YCL076W	ty fragment	YDR426C	YDR425W	YGR259C	TNA1
YCR013C	PGK1	YDR431W	YDR430C	YGR265W	MES1
YCR018C-A	LTR	YDR433W	NPL3	YGR290W	MAL11
YCR041W	MATALPHA1	YDR442W	SSN2	YHR125W	LTR
YCR049C	ARE1	YDR445C	YDR444W	YHR145C	LTR
YCR050C	ARE1	YDR455C	NHX1	YHR214W-A	YHR214
YCR064C	YCR063W	YDR467C	YDR466W	YIL060W	delta LTR
YCR087W	YCR087C-A	YDR509W	YDR509C	YIL080W	YIL082W
YDL009C	YDL010W	YDR521W	YDR520C	YJL007C	tRNA AND LTR
YDL011C	YDL010W	YDR526C	YDR527W	YJL009W	CCT8
YDL016C	CDC7	YDR537C	PAD1	YJL018W	YJL019W
YDL023C	GPD1	YEL076C-A	YELO76C	YJL022W	PET130
YDL026W	YDL027C	YEL076W-C	YEL077C	YJL032W	BET4
YDL032W	YDL033C	YER066C-A	YER067W	YJL075C	NET1

Table 2. *Continued*

Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature
YJL086C	YJL087C	YLR261C	YPT6	YNL170W	PDS1
YJL119C	YJL118W	YLR279W	YLR281C	YNL171C	APC1
YJL142C	YAK1	YLR280C	YLR281C	YNL174W	NOP13
YJL152W	YJL151C	YLR282C	YLR283W	YNL184C	MRPL19
YJL169W	SET2	YLR317W	TAD3	YNL203C	SPS19
YJL175W	SWI3	YLR322W	SFH1	YNL205C	SPS18
YJL188C	RPL39	YLR331C	MID2	YNL226W	YNL227C
YJL195C	CDC6	YLR334C	LTR	YNL228W	YN227
YJL202C	PRP21	YLR338W	VRP1	YNL235C	SIN4
YJL211C	PEX2	YLR339C	RPP0	YNL266W	IST1
YJL220W	FSP2	YLR349W	DIC1	YNL276C	MET2
YJR018W	ESS1	YLR358C	RSC2	YNL285W	LTR and Trna
YJR020W	TES1	YLR374C	STP1	YNL296W	MON2
YJR023C	LSM8	YLR379W	SEC61	YNL319W	HXT14
YJR037W	HUL4	YLR428C	CRN1	YNL324W	FIG4
YJR038C	YJR039w	YLR434C	YLR435W	YNR005C	VPS27
YJR071W	YJR070C	YLR444C	ECM7	YNR042W	COQ2
YJR079W	YJR080C	YLR458W	NBP1	YOL013W-A	LTR
YJR087W	YJR088C	YLR463C	YRF1-4	YOL035C	YOL036W
YJR128W	ZMS2	YLR465C	YRF1-4	YOL037C	YOL036W
YKL030W	MAE1	YML010C-B	SPT5	YOL046C	YOL045W
YKL036C	YKL037W UGP1	YML010W-A	SPT5	YOL050C	GAL11
YKL053W	YKL052C	YML013C-A	YML013W	YOL079W	REX4
YKL076C	YKL075C	YML035C-A	SRC2	YOL099C	PKH2
YKL083W	YKL082C	YML048W-A	PRM6	YOL106W	LTR
YKL111C	ABF1	YML058C-A	CMP2	YOL134C	HRT1
YKL118W	VPH2	YML095C-A	GIM5	YOL150C	GRE2
YKL123W	SSH4	YML100W-A	ARG81	YOR053W	YOR054C
YKL131W	RMA1	YML102C-A	CAC2	YOR055W	YOR054C
YKL136W	ALP2	YML117W-A	YML117W	YOR068C	new VPS5
YKL147C	YKL146W	YMR046W-A	LTR	YOR082C	YOR083W
YKL153W	GPM1	YMR052C-A	FAR3 and STB2	YOR102W	OST2
YKL169C	MRPL38	YMR075C-A	YMR075W	YOR105W	YOR104W
YKL177W	STE3	YMR119W-A	YMR119W	YOR121C	GCY1
YKL202W	MNN4	YMR135W-A	YMR135C	YOR135C	IDH2
YKR012C	PYR2	YMR153C-A	NUP53	YOR139C	SFL1
YKR033C	DAL80	YMR158C-B	LTR	YOR146W	YOR145C
YKR035C	FTI1	YMR158W-A	APG16	YOR169C	GLN4
YKR047W	NAP1	YMR172C-A	HOT1	YOR170W	LCB4
YLL020C	KNS1	YMR173W-A	DDR48	YOR200W	PET56
YLL037W	PRP19	YMR193C-A	YMR194W	YOR203W	HIS3 and DED1
YLL044W	RPL8B	YMR244C-A	YMR245W	YOR218C	RFC1
YLL047W	RNP1	YMR290W-A	HAS1	YOR225W	ISU2
YLR041W	YLR040C	YMR294W-A	YMR295C	YOR248W	SRL1
YLR062C	RPL22-A	YMR306C-A	FKS3	YOR263C	YOR264W
YLR076C	RPL10	YMR316C-A	DIA1	YOR277C	CAF20
YLR101C	ERG27	YMR316C-B	YMR317W	YOR282W	PLP2
YLR140W	RRN5	YNL013C	YNL014W	YOR300W	BUD7
YLR169W	APS1	YNL017C	Trna	YOR309C	NOP58
YLR171W	APS1	YNL043C	YIP3	YOR325W	YOR324C
YLR198C	SIK1	YNL057W	YNL058C	YOR331C	VMA4
YLR202C	YLR201C	YNL089C	RHO2	YOR333C	MRS2
YLR217W	CPR6	YNL105W	INP52	YOR345C	REV1
YLR230W	CDC42	YNL109W	YNL108C	YOR364W	YOR365C
YLR232W	YLR231C	YNL114C	RPC19	YOR366W	YOR365C
YLR235C	TOP3	YNL120C	YNL119W	YOR379C	YOR378W
YLR252W	YLR251W	YNL140C	RLR1	YPL035C	YPL034W

Table 2. *Continued*

Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature	Spurious ORF	Overlapping feature
YPL044C	NOP4	YPL251W	YAH1	YPR087W	SRP54
YPL073C	UBP16	YPR002C-A	LTR	YPR099C	YPR0100W
YPL102C	YPL101W	YPR038W	YPR037C	YPR123C	CTR1
YPL114W	YPL113C	YPR039W	YPR037C	YPR126C	YPR125W
YPL136W	YPL137W	YPR044C	RPL43A	YPR130C	SCD6
YPL142C	RPL33A	YPR050C	MAK3	YPR136C	RRP9
YPL182C	YPL181W	YPR053C	NHP6A	YPR177C	PRP4
YPL185W	YPL186c	YPR059C	YMC1	YPR197C	SGE1
YPL197C	RPL7B	YPR076W	OPY2		
YPL238C	SUI3	YPR077C	YPR078C		

significant, but spurious, TBLASTX matches to alternative frames of the real gene (anti-sense or sense-different reading frame).

Of the ORFs previously defined as questionable but now proposed to be coding by Malpertuy *et al.* (2000), and retrievable from the Genolevures website (**http://cbi.labri.u-bordeaux.fr/Genolevures/Genolevures.php3**), at least 107 out of 136 occur in overlapping pairs. These pairs have two significant TBLASTX hits when the ascomycete DNA is compared to the *S. cerevisiae predicted* protein set; the best score belonging to the real coding sequence and a lower score generated by the overlap with the spurious ORF and an alternative translation of the closely related organism's DNA. This is illustrated by the three pairs of overlapping genes *YGR220C*/*YGR219w*, *YOR054c*/*YOR055w*, and *YDR443C*/*YDR442w* in Table 4 (data from the Genolevures website). The correct reading frame should also be apparent if levels of synonymous and nonsynonymous nucleotide substitution are calculated for the aligned regions.

After the merging of sequences identified in *Merged and extended genes* (Table 1), only eight genes of known or inferred function in the entire *S. cerevisiae* genome remain overlapping. The overlaps and their orientations are listed in Table 5. The longest overlaps observed were 55 and 34 amino acids, which are possibly attributable to sequencing anomalies, or deletions; the other six are 10 amino acids or less, and predominantly C-terminal.

Overlapping CDS features are also rare in *Sz. pombe*. Of 4189 genes which are characterised or conserved, only three pairs have an overlap greater than 10 amino acids in length, none of which were at the N terminus. Moreover, since the completion of the *S. cerevisiae* genome, no function or biologically significant similarity to any other sequenced organism has been observed for any of the largely overlapping ORFs designated here as spurious. This is despite the major efforts of EUROFAN and other functional genomics studies to determine the function of every yeast gene, and the exponential increase in protein sequences deposited in the public databases.

Considering the rarity of overlapping genes in both yeasts, and the absence of unequivocal functional evidence in support of the coding integrity of any of the spurious ORFs which are wholly or largely overlapping real genes, the likelihood that any encode for proteins is minimal. Therefore, it would be prudent to remove them completely from the genome totals and label them accordingly in the public databases.

Very hypothetical proteins

One advantage of discriminating between sequence orphans likely to be coding, and *very hypothetical* orphans, is that these regions of DNA can be easily partitioned as a subset, facilitating the identification of other features by bioinformatics analyses.

Despite over 1000 *Sz. pombe* experimental gene characterisations, only three of the 177 *Sz. pombe* ORFs annotated as '*very hypothetical protein*' have so far been shown to be protein coding. One of these, git11, is a 76 amino acid protein, and is below the threshold imposed on length, but appeared to have coding potential. The size distribution and splicing frequency of *Sz. pombe* orphans, when compared to genes of known or inferred function, suggests that a larger proportion may not be real (manuscript in preparation).

Table 3. Very hypothetical proteins with no homology and low coding potential

CDS	CDS	CDS	CDS
YAL069W	YEL045C	YIL100W	YLR385C
YAL066W	YEL033W	YIL086C	YLR400W
YAR029W	YEL010W	YIL054W	YLR402W
YAR030C	YER053C-A	YIL025C	YLR415C
YAR047C	YER066C-A	YIL012W	YLR416C
YAR053W	YER071C	YIL059C	YLR184W
YAR060C	YER084W	YIL058W	YML108W
YAR064W	YER091C-A	YIL032W	YML090W
YAR069C	YER092W	YIR020C	YML089C
YAR070C	YER093C-A	YIR020W-B	YML084W
YBL071C	YER097W	YIR044C	YMR031W-A
YBL048W	YER121W	YJL199C	YMR151W
YBL129C-A	YER181C	YJL182C	YMR057C
YBR012C	YER187W	YJL150W	YMR086C-A
YBR013C	YER189W	YJL135W	YMR194C-A
YBR027C	YFL015C	YJL120W	YMR245C
YBR032W	YFL019C	YJL027C	YMR304C-A
YBR209W	YGL261C	YJL015C OR YJL016W	YMR320W
YBR300C	YGL260W	YJR114W OR YJR113C	YMR324C
YBR134W	YGL204C	YJR146W	YNL338W
YBR178W	YGL193C	YJL067W OR YJL066C	YNL337W
YBR190W	YGL188C	YJL065C OR YJL064W	YNL303W
YCL021W-A	YGL182C	YJL062W-A	YNL300W
YCR006C	YGL177W	YJL052-A	YNL226W
YCR022C	YGL149W	YKL162C-A	YNL198C
YCR025C	YGL052W	YKL102C	YNL179C
YCR043C	YGL051W	YKL044W	YNL149C OR YNL150W
YCR085W	YGL041C	YKL031W	YNL146W
YCR102W-A	YGR025W	YKL115C	YNL028W
YDL242W	YGR050C	YKR032W	YNR025C OR YNR024W
YDL196W	YGR051C	YKR041W OR YKR040C	YNR001W-A
YDL172C OR YDL173W	YGR069W	YKR073C	YOL166C
YDL162C	YGR139W	YKL106C-A	YOL026C
YDL157C	YGR182C	YKL018C-A	YOR012W OR YOR013W
YDR114C	YGR269W	YLL030C	YOR024W
YDR029W	YGR291C	YLR111W	YOR041C OR YOR042W
YDR157W	YGR293C	YLR112W	YOR225W
YDR209C	YGR161W-A	YLR123C OR YLR122C	YOR235W
YDR220C	YGR271C-A	YLR161W	YOR268C
YDR320C-A	YHL045W	YLR162W	YOR304C-A
YDR504C	YHL005C	YLR255C	YOR343C
YDR491C	YHR217C	YLR269C	YPL261C
YDR034W-B	YHR130C	YLR294C	YPL205C
YDR070C	YHR139C-A	YLR311C	YPR012W
YEL074W	YHR173C	YLR302C	YPR092W
YEL073C	YIL174W	YLR346C	YPR142C OR YPR143W
YEL068C	YIL163C	YLR365W	YPR146C
YEL059W	YIL141W	YLR366W	YPR150W OR YPR151C
YPR074W-A			

Implications for post genomics

Many of the spurious overlapping ORFs included in the public databases, and proposed as disregarded ORFs by this analysis, have associated functional genomics data which could be artefacts. The original yeast microarrays (using PCR products), were not strand specific with respect to the probes (DeRisi *et al.*, 1997), and opposite strand transcripts could hybridise to these array spots (D. Vetrie, perscomm).

Table 4. Real/false pairs

Ascomycete DNA	Real gene	Length	P value	Blast Score	Disregarded ORF	Length	P value	Blast Score	Overlap Length
AR0AA003F06TP1	YGR220C MRPL9	269	7e-69	72	YGR219w	113	0.01	31	100
AR0AA005F12TP1	YOR054c	674	2e-09	52	YOR055w	144	2e-10	49	127
AR0AA008C07CP1	YDR443C SSN2	1420	2e-16	130	YDR442w	130	3e-12	66	120

Positive signals may also result from overlapping UTRs. Not unexpectedly, many of the disregarded ORFs have transcript profiles similar to the overlapping characterised gene. Gene knockouts of spurious ORFs may give phenotypes, particularly if they affect overlapping strand ORFs, promoters, or other cellular RNAs. It has been observed that some of the knockouts of overlapping ORFs have essentially the same, or similar phenotype to the real adjacent gene. These transcript and phenotype artefacts, attached to the database entries, lend these predictions false credibility as proteins. The inclusion of spurious ORFs may therefore affect the accuracy of any previous global analysis of transcription or redundancy.

New gene number estimate

Our analysis provided a new estimate of gene number for each *S. cerevisiae* chromosome. These are provided in Table 6.

When *S. cerevisiae* was first published, 6275 ORFs were predicted; 390 of these were proposed to be spurious giving a probable gene number of 5885 (Goffeau *et al*., 1996). The data used for our analysis (SGD) consisted of 6282 ORFs, of which 370 have been disregarded, giving a new *maximum* upper limit of 5804. The removal of 42 pseudo or frame-shifted sequences, and 193 very hypothetical proteins further reduces this total to 5570. This is likely to be closer to the true upper limit, because the criteria used for the determination of very hypothetical proteins are quite conservative. There is a possibility that a small number of the very hypothetical proteins may eventually be determined to be coding, but size distribution (unpublished) indicates that we may still be over estimating the number of small ORFs.

Malpertuy *et al.* predicted a gene number of 5651. In addition, using two different statistical methods, they estimated that the actual number of protein coding ORFs should be either 5542 or 5552, but do not account for the differences between their predicted number of 5651 and the statistical calculations. The statistical calculations are closer to the number of genes predicted by our analysis (5570 or fewer). The discrepancies could be due to the inclusion of novel genes which are in fact spurious (see Discussion, Novel Genes), or the inclusion of genes previously defined as questionable, but proposed by this analysis to be disregarded (see Discussion, Spurious ORFs).

Table 5. Real *S. cerevisiae* genes with overlaps

Gene 1	Gene 2	Overlap (AA)	Orientation
BUD5	MATALPHA2	0.3	N/C
AUA1	YFL010C	55	C/C
AMD1	PRP38	1	C/C
ECM12	YHR022C	34	C/C
VPS38	YLR361C	10	C/C
YML096W	RAD10	1	C/C
YNL246W	YNL245C	0.3	C/C
CTF19	YPL017C	8	C/C

N = N terminus, C = C terminus, AA = Amino acid length.

What are the remaining orphans?

Data obtained by Gaillardin *et al.* (2000) demonstrated that ascomycete specific genes are highly represented in the functional classes of *cell wall organisation*, *extracellular/secreted proteins*, and *transcriptional regulators* suggesting that they diverge more rapidly than other classes of genes. In *Sz. pombe*, many remaining orphans are low complexity or repetitive proteins e.g. serine-rich with low similarity to alpha-agglutins and other cell surface proteins, or proteins with basic charged regions which may correspond to transcription factors. It may be that most orphans correspond to genes which have diverged so much that they are unrecognisable, rather than novel genes. It is therefore possible that the majority of orphans are genes which have diverged more rapidly and that the number of truly species specific genes is very small.

A comparison of the refined orphan sets of

Table 6. Predicted *S.cerevisiae* gene numbers, by chromosome

Chromosome number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	Total
ORF No. (Goffeau *et al.* 1996)	110	422	172	812	291	135	572	288	231	387	334	547	487	421	569	497	6275
Questionable (Goffeau *et al.* 1996)	3	30	12	65	13	5	57	12	11	29	20	41	30	23	3	36	390
SGD 2000 ORFs	107	428	173	819	288	134	571	284	224	387	335	547	491	421	573	500	6282
Spurious non coding	8	27	16	61	7	3	39	3	2	25	19	38	27	27	38	28	368
Sanger new	98	397	154	743	275	122	525	275	215	354	317	497	455	391	527	459	**5804**
Pseudo or frameshift	2	2	3	1	2	2	2	3	5	5	2	4	3	1	3	2	42
Sanger very hypothetical	10	13	6	16	16	1	21	7	12	14	11	19	12	13	11	10	192
Final	86	382	145	726	257	119	502	265	198	335	304	474	440	377	513	447	**5570**

Sz. pombe and *S. cerevisiae* will aid the detection of subtle homologies and physical similarities between the sequence orphans themselves, or orphans and previously characterised genes. For example, the final subunit of *Sz. pombe* RNA polymerase III (the homologue of *S. cerevisiae* RPC31) was identified due to similarity in amino acid length and the presence of an acidic C terminus, despite a low similarity score (Richard Maraia, NICHD, NIH, Bethesda and George Shpakovski, Russian Academy of Sciences, Moscow. pers comm).

Global comparison of the remaining orphans will facilitate definition of the sets of genes necessary for unicellular eukaryotic life. However, to do this effectively, it is important that a distinction is first made between orphans and spurious ORFs (Malpertuy *et al.*, 2000).

Conclusions

A substantial proportion of small orphans are probably not protein coding, yet may define other genome features (regulatory regions, cellular RNAs or even gene-free regions which may be involved in higher order chromosome structure). These may contain spurious ORFs which, if defined as CDS, appear to generate matches at the protein level. Spurious gene predictions, with associated artefactual functional genomics data, will exclude these regions of DNA from being inspected for non-CDS features. Attaching a suitable annotation to these would facilitate the detection of authentic features.

It is important to differentiate firstly between orphans and disregarded spurious ORFs, and secondly, between likely real orphans and very hypothetical orphans. Refinement of the orphan sets of sequenced genomes will enable the detection of more subtle homologies and other physical similarities between the real orphans.

As the number of orphans is gradually eroded by the removal of non-coding ORFs and the detection of distant homologues, it will become easier to determine how many truly species specific genes exist in the *Sz. pombe* and *S. cerevisiae* genomes.

The annotation of an ORF's status within the public datasets is important for both functional genomics and bioinformatics. The costs of reagents, labour and curation of 370 ORFs which should be disregarded in functional genomics analyses are not trivial, they account for roughly 5% of the total effort. Bioinformatics on proteome data to examine amino acid composition, charge, etc. require accurate datasets (perhaps with different confidence levels attributed). Integration of contextual information on a gene-by-gene basis to determine status will enable the targeting of future research toward genes which are more likely to be coding.

As more analyses are performed, we should get closer to the absolute gene number. Taken in combination, previous analyses and the interpretion of the biological context of the ORF should enable better estimates of probable gene and orphan number for this yeast.

Data availability

Updated EMBL format sequences (containing nearly 12000 annotations) which can be examined in Artemis and a one-gene one-protein FASTA format protein translations database are available from the Sanger Centre ftp site (ftp://ftp/pub/yeast/SCreannotation).

The EMBL entries will continue to be maintained (and will be resubmitted to EMBL with permission

of the original authors). Further refinement of the datasets described will include:

1. New similarity information from BLASTX.
2. EST/cDNA mappings.
3. Regulatory region identification/mapping.
4. Inclusion of other annotated features (keys and qualifiers) from individual GenBank/EMBL entries.

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