

De-Novo Assembly and Analysis of the Heterozygous Triploid Genome of the Wine Spoilage Yeast *Dekkera bruxellensis* AWRI1499

Chris D. Curtin^{1*}, Anthony R. Borneman¹, Paul J. Chambers¹, Isak S. Pretorius^{1,2}

¹ The Australian Wine Research Institute, Adelaide, Australia, ² The University of South Australia, Adelaide, Australia

Abstract

Despite its industrial importance, the yeast species *Dekkera (Brettanomyces) bruxellensis* has remained poorly understood at the genetic level. In this study we describe whole genome sequencing and analysis for a prevalent wine spoilage strain, AWRI1499. The 12.7 Mb assembly, consisting of 324 contigs in 99 scaffolds (super-contigs) at 26-fold coverage, exhibits a relatively high density of single nucleotide polymorphisms (SNPs). Haplotype sampling for 1.2% of open reading frames suggested that the *D. bruxellensis* AWRI1499 genome is comprised of a moderately heterozygous diploid genome, in combination with a divergent haploid genome. Gene content analysis revealed enrichment in membrane proteins, particularly transporters, along with oxidoreductase enzymes. Availability of this assembly and annotation provides a resource for further investigation of genomic organization in this species, and functional characterization of genes that may confer important phenotypic traits.

Citation: Curtin CD, Borneman AR, Chambers PJ, Pretorius IS (2012) De-Novo Assembly and Analysis of the Heterozygous Triploid Genome of the Wine Spoilage Yeast *Dekkera bruxellensis* AWRI1499. PLoS ONE 7(3): e33840. doi:10.1371/journal.pone.0033840

Editor: Cecile Fairhead, Institut de Genetique et Microbiologie, France

Received: November 8, 2011; **Accepted:** February 17, 2012; **Published:** March 28, 2012

Copyright: © 2012 Curtin et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Research at the Australian Wine Research Institute is funded by Australia's grapegrowers and winemakers through their not-for-profit investment body, The Grape and Wine Research Development Corporation, with matching funding from the Australian government. Omics and Systems biology research at the AWRI uses resources provided as part of the National Collaborative Research Infrastructure Strategy (NCRIS), an initiative of the Australian Government, in addition to funds from the South Australian State Government. AWRI's collaborating partners within this NCRIS-funded initiative—which is overseen by Bioplatforms Australia—are Genomics Australia, Proteomics Australia, Metabolomics Australia (of which the Microbial Metabolomics unit is housed at the AWRI) and Bioinformatics Australia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: chris.curtin@awri.com.au

☯ These authors contributed equally to this work.

Introduction

The yeast species *Dekkera (Brettanomyces) bruxellensis* is predominantly associated with wine, beer, and cider, but has also been detected in kombucha (sweet tea) and sourdough fermentations [1,2]. In light of this association with fermentation environments, it is unsurprising that recent studies have highlighted the potential for this yeast species to be harnessed in industrial bio-ethanol production processes [3–5].

The nearest phylogenetic neighbour of *D. bruxellensis* is *Dekkera anomala*, a species that has been found to co-exist with *D. bruxellensis* in some niches, such as cider fermentation [6], but has never been unambiguously isolated from wine [7]. *D. bruxellensis* is well adapted to this environment. Some of these adaptations; osmotolerance, ethanol tolerance, acid tolerance, are shared with *Saccharomyces cerevisiae* - the ubiquitous fermentation yeast that precedes *D. bruxellensis* in population ecology of the industrial fermentation systems mentioned above. In addition, *D. bruxellensis* is both petite and crabtree positive, and has convergently evolved the make-accumulate-consume lifestyle in parallel with *S. cerevisiae* [8]. However, *D. bruxellensis* is capable of surviving and proliferating for extended periods of time after *S. cerevisiae* has completed alcoholic fermentation and its population declined [9].

It is well accepted that *D. bruxellensis* is the key wine microorganism that converts hydroxycinnamic acids present in

grape must and wine into the volatile phenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) and 4-ethylcatechol (4-EC). Wine containing above-threshold concentrations of these compounds exhibits 'Brett' character [10], which can result in wines that are less-preferred by consumers [11]. Spoilage of wine by *D. bruxellensis* is, in fact, considered the foremost microbiological issue in the wine industry [12]. In some industries the presence of *D. bruxellensis* is desirable. For example, the Belgian lambic and gueuze ales are typified by flavours generated during secondary fermentation by this yeast [13] through production of the same volatile compounds (4-EP and 4-EG). This dichotomy is in part due to the difference in relative concentration of these volatile phenols - beer generally contains higher concentrations of 4-EG (clove-like, or spicy aroma), while wine contains more 4-EP (medicinal aroma).

Partial survey sequencing of the *D. bruxellensis* CBS 2499 genome [14] revealed an enrichment of genes encoding transporters and enzymes associated with nitrogen and lipid metabolism, which may go some way to explaining the capacity of this yeast to survive in the relatively barren post-alcoholic fermentation environment. However, due to the incomplete nature of these data, the genomic organization for this species is less certain. Re-analysis revealed the previously assumed haploid status for CBS 2499 was incorrect, and instead it appeared to be a relatively heterozygous diploid/polyploid [15]. Extensive karyotype vari-

ability was also observed among *D. bruxellensis* strains in the same study, leading the authors to question existence of a sexual cycle for this species.

Despite its importance in food and beverage industries, very little is known regarding the genetics of this microorganism. Next-generation genomic sequencing is providing the means of fast tracking genetic analysis of intractable, non-model organisms, by enabling direct access to genetic make-up, and subsequent functional analysis. To gain insight into the genome features and gene content of *D. bruxellensis*, we have sequenced the prevalent wine spoilage strain, AWRI1499 [7]. We found *D. bruxellensis* AWRI1499 has a triploid genome, that relative to phylogenetically related species is enriched in genes that may aid survival in the challenging environment of wine.

Results and Discussion

Genomic assembly and annotation

De-novo auto-assembly of sequence reads yielded 26,043 contigs, with N50 of 7 kb at 9.7-fold coverage, and an assembly length of 40.2 Mb. The genome size of *D. bruxellensis* was estimated at between 20 and 30 Mb by pulsed-field electrophoresis of several European strains [14]. This estimation, in combination with paired-end information present in our assembly suggested that many small contigs had not been incorporated into larger contigs by the assembler due to heterozygosity. Manual editing of the assembly was therefore used to reassemble contigs into larger scaffolds under the assumption that AWRI1499 was diploid. Manual finishing yielded a 12.7 Mb assembly comprised of 324 contigs (N50 = 68 kb) in 99 scaffolds, at median coverage of 26-fold (see Table S1 for contig statistics). ‘Unlocated’ contigs remaining after this process that were >500 bp in length, and consisted of at least 10 sequence reads, were found to harbour at least 22 Ty3-gypsy-like, and 43 Ty1-copia-like transposable elements. AT-rich contigs AWRI1499 with high read depth were designated as putative mitochondrial genome sequences and separated from the genomic assembly. Assembly of these contigs against the mitochondrial genome recently published for *D. bruxellensis* CBS 2499 [16] resulted in almost complete coverage (99.7%) and high levels of nucleotide identity (>98% across 20 kb alignments) (data not shown). Average coverage for these contigs was 225-fold, suggesting approximately nine copies of the mitochondrial genome per *D. bruxellensis* cell.

RNA samples pooled from cultures grown under standard laboratory conditions, and in the presence of 2 mM ferulic acid [17], were sequenced and mapped to the genomic assembly to provide information on gene positions. Gene model prediction was conducted using Augustus [18], taking exon hints based upon this mapped RNAseq data, and predicted proteins annotated using Blast2go [19]. Of the 4969 gene models predicted by Augustus, 688 were not assigned a significant match by Blast2go against the NCBI non-redundant protein database. Median length of unannotated gene models was 531 nucleotides, compared with 957 nucleotides for annotated gene models. When translated, 14.5% of gene models lacking annotation yielded proteins shorter than 100 amino acids, and may therefore be considered spurious. Consequently, there are 588 gene models in this assembly coding for orphan proteins, making up 11.8% of *D. bruxellensis* AWRI1499 predicted proteins.

To test the AWRI1499 genome assembly for completeness, the core eukaryote gene (CEG) set of 248 proteins from six reference species [20] were used as translated nucleotide blast (tblastn) queries against the AWRI1499 predicted open reading frames (ORFs). 213 CEG proteins were detected (E-values < 1e-20), while

a further 20 CEGs were found by tblastn against the AWRI1499 scaffold assembly, suggesting the Augustus gene model prediction failed to detect around 10% of potential ORFs. Four CEGs (corresponding to *S. cerevisiae* ORFs YGR181w, YOR103c, YGL231c, YGL047w) were detected at low identity or had only partial matches in the scaffold consensus. A further 11 CEGs with AWRI1499 gene model tblastn hits at worse than >1e-20 were confirmed by tblastn of non-*S.cerevisiae* fungal orthologs of each protein against the scaffold consensus. This analysis suggested the genomic assembly of AWRI1499 was approximately 98% complete.

Phylogenetic position of *D. bruxellensis* AWRI1499

The 20 fungal species most represented as blastp top-hits for AWRI1499 gene models (Figure S1) were selected for phylogenetic analysis. Protein sequences were obtained for each species and pair wise protein blasts performed. A total of 542 putative orthologous proteins present in all species (as defined by E-values of less than 1e-20) were aligned separately and then concatenated into a single hybrid sequence, from which a maximum-likelihood phylogeny was constructed (Figure 1). The topology of this phylogeny reflected the top-hit statistics (Figure S1), with *Pichia angusta* the closest relative to *D. bruxellensis* among those fungi for which sequenced genomes are available. Along with *Pichia pastoris*, these species, while not closely related, form a grouping distinct from the whole genome duplication and CTG clades, respectively.

SNP density and haplotype sampling

Significant heterozygosity was observed in the assembly, with a median of 27 single nucleotide polymorphisms (SNPs) per 1000 nucleotides - a total of 342,900 sites across the genome. However, this density was not uniform across the assembly with some regions displaying substantially reduced rates of heterozygosity (Figure 2). In particular, contigs comprising scaffold 3 of the assembly displayed a significantly reduced SNP density.

Within predicted open reading frames there were fewer SNPs than seen for the entire genome, with an average of 1.9%. The majority of these coding region SNPs were synonymous. Nonetheless 0.66% of all nucleotides are predicted to produce non-synonymous amino acid substitutions across *D. bruxellensis* ORFs. During assembly it was noted that in many regions more than two distinct sequences were evident amongst sequence reads. To investigate the distribution of coding region SNPs across multiple alleles, a subset of ORFs (60 of 4969) with very high SNP density (6%), high SNP density (4%), and moderate SNP density (2%) were randomly sampled across the genome, and manually separated into haplotypes. Of the 60 ORFs sampled, 37 could be resolved into three distinct haplotypes. ORFs from which two haplotypes were resolved did not originate only from regions with low SNP density, such as scaffold 3, but were dispersed across the assembly (data not shown). The probability of resolving three haplotypes from ORFs with 2% SNP density was, however, significantly lower (P = 0.0147, Pearson Independence Test). Pair wise nucleotide sequence alignments (Figure 3) revealed that for most loci two haplotypes were closely related (~99% identity), while the third displayed significantly greater sequence divergence (~95% identity). Nucleotide identity at loci where only two haplotypes were recovered was found to span a range from 91–99%, indicating that a similar level of overall sequence divergence was captured in these two haplotypes as was found in those that could be separated into three distinct alleles. This genome assembly does not permit haplotype phasing, however the most parsimonious explanation for the presence of three alleles for these genes (two closely related and a third more diverged) would be that

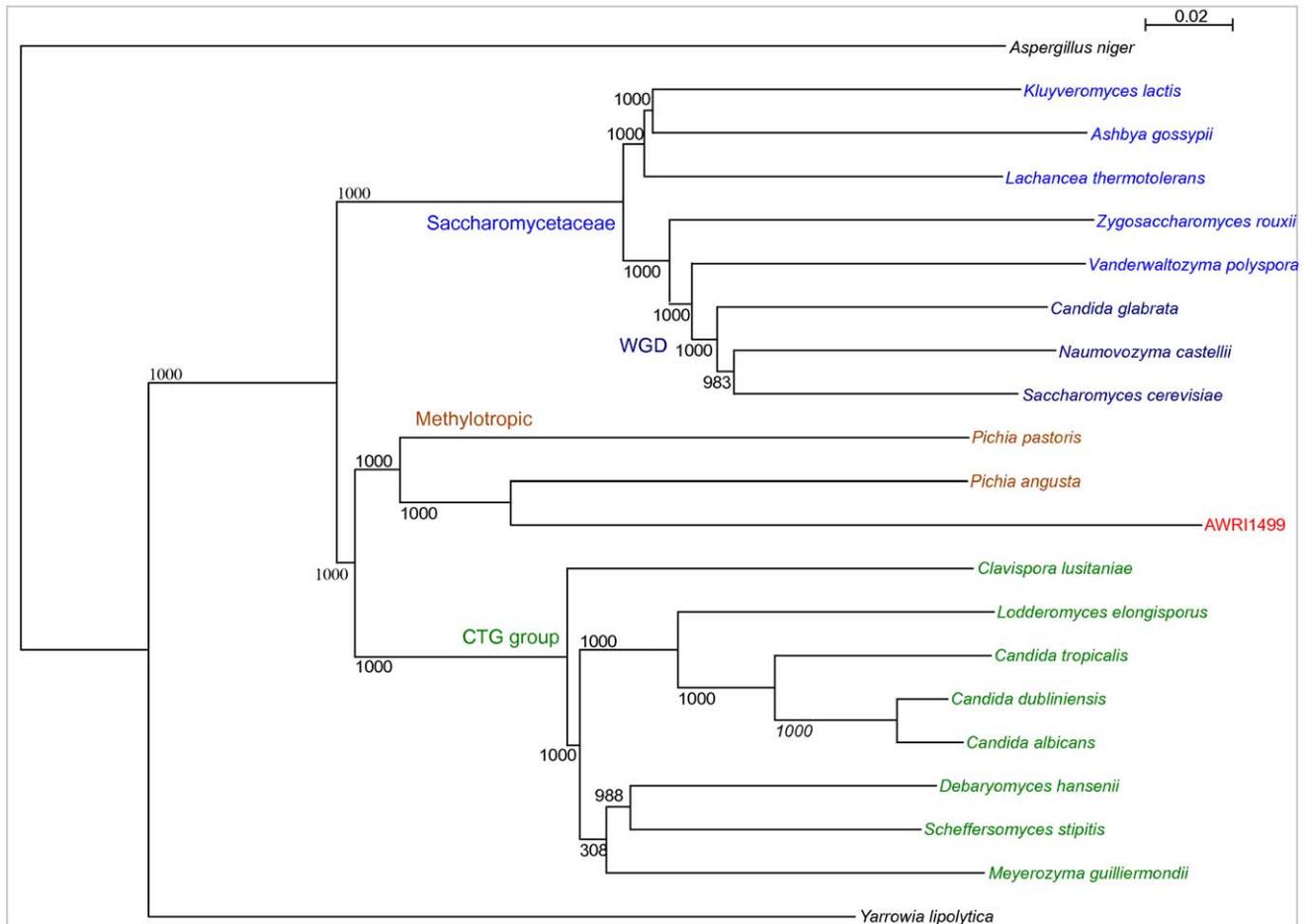


Figure 1. Phylogeny for species most represented in blastp searches for *D. bruxellensis* AWRI1499 proteins. A Maximum likelihood tree was generated from an amino-acid alignment of 542 putative orthologous proteins. Bootstrap values (1000 randomisations) for all nodes shown. doi:10.1371/journal.pone.0033840.g001

the genome of *D. bruxellensis* AWRI1499 comprises a moderately heterozygous diploid genome, in addition to a divergent haploid genome. In combination with observations for a very limited number of genes from other strains of *D. bruxellensis* [15], this data indicates that *D. bruxellensis* arose through the hybridisation of two closely related species (one diploid, one haploid). Regions of reduced SNP density, such as scaffold 3, would therefore most likely reflect a partial replacement of the divergent haploid by the diploid genome, a phenomenon observed in *Saccharomyces* interspecific hybrids [21]. Furthermore, mechanisms that drive genome stabilisation in such hybrids [22] could explain the extreme karyotypic variability observed for *D. bruxellensis* [15]. Whilst the triploid status of *D. bruxellensis* does not by default infer sterility [23], along with the observation of extreme karyotypic variability displayed by this species [15], it is likely that *D. bruxellensis* lacks a fully functional sexual cycle.

Gene content analysis

To ascertain if there were any genes which could provide insight into the evolutionary history of *D. bruxellensis*, the predicted proteome of AWRI1499 was submitted to OrthoMCL for assignment to orthologous clusters (Table S2), along with the proteomes of *P. angusta*, *P. pastoris*, and *S. cerevisiae*. While the two *Pichia* strains were included for analysis due to their evolutionary relationship to *D. bruxellensis*, *S. cerevisiae*, which is distantly related

to *D. bruxellensis*, was included as these two species occupy very similar environmental niches (alcoholic fermentation) and are hypothesised to have undergone convergent evolution of several phenotypic traits [8]. Figure 4 summarises the number of OrthoMCL clusters shared between species. Within the 175 clusters unique to *D. bruxellensis* were 198 predicted proteins. Seventy-seven of these proteins were not annotated by Blast2go which has a more stringent blastp cut-off ($E < 1e-6$). A further 64 were found to have a blastp match to a protein from one of the other three species (assigned to a different OrthoMCL cluster) and there were 10 dubious assignments of short predicted proteins (<100 amino acids). Excluding four transposon-like proteins, there were no significantly enriched GO-terms for the remaining 43 proteins (Fisher's exact test, FDR 0.05). Nonetheless, some of these may contribute to the 'scavenging' lifestyle of this yeast. Several proteins involved in carbon source utilisation (chitin, *N*-acetylglucosamine, galactose, mannose, lactose), were detected. Interestingly, most genes required for chitin/*N*-acetylglucosamine catabolism were found to be present in a single cluster of five genes, which were also found to be present in *M. guilliermondii*, although separated in two blocks of synteny (Figure 5). This pathway may represent efficient recycling of cell wall building blocks when required, or provide access for *D. bruxellensis* to an abundant carbon source in fungal communities.

To detect instances of conserved gene family expansion during the evolution of *D. bruxellensis*, a total of 2,317 OrthoMCL clusters

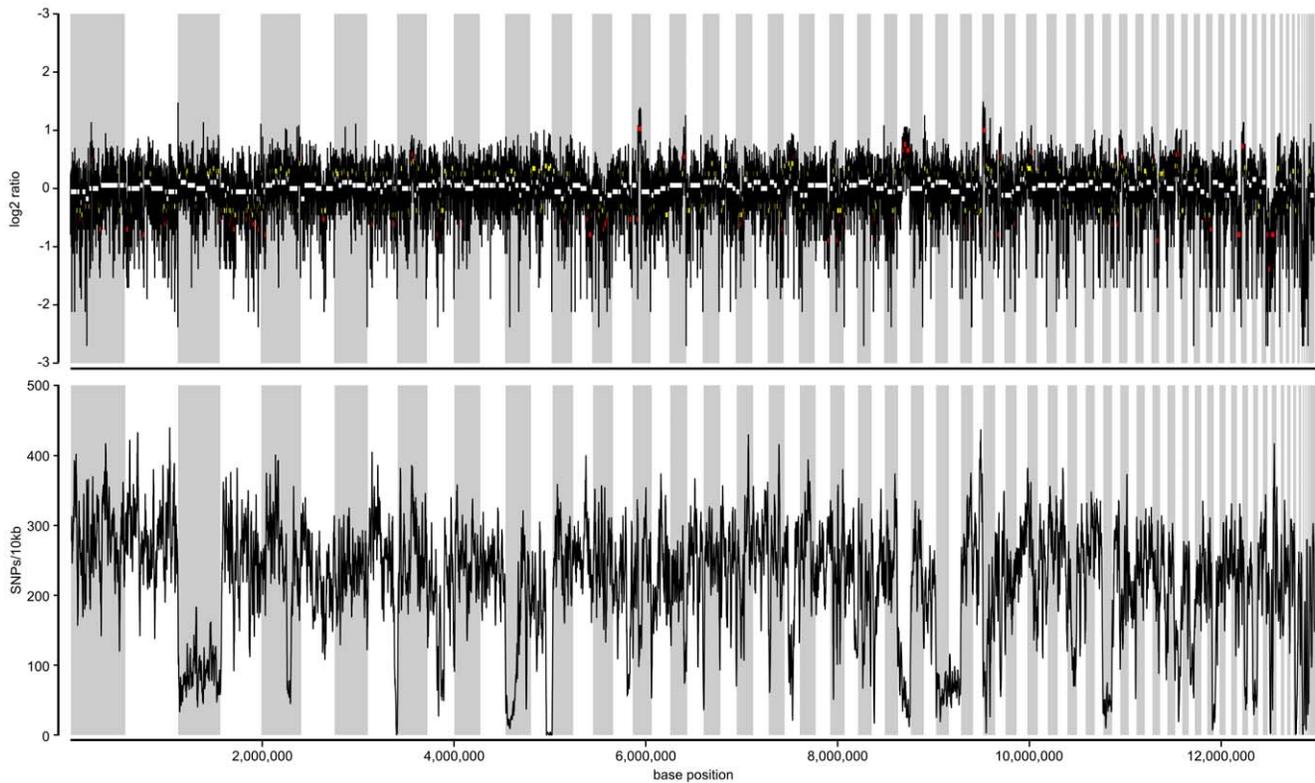


Figure 2. Sequencing coverage and SNP density of *D. bruxellensis* AWRI1499 genomic assembly. Coverage (log₂ ratio of overall median) and SNP density (per 10,000 nucleotides) determined for each contig using a sliding window of 1001 bp, with a 100 bp step frequency, and plotted in scaffold order. Scaffold boundaries are indicated by alternate shading, regions of copy number variation greater than 1.25- or 1.5fold indicated by yellow, and red dots, respectively. White dots indicate coverage consistent with overall genomic median.
doi:10.1371/journal.pone.0033840.g002

that were represented by at least one protein in all four species were examined for either: expansion specifically in *D. bruxellensis*, lineage-specific expansion (expansion relative to *S. cerevisiae* but not *P. angusta* or *P. pastoris*) and expansion driven by convergent

evolution (expansion relative to *P. angusta* and *P. pastoris*, but not *S. cerevisiae*). *D. bruxellensis* proteins corresponding to expanded clusters were then examined for GO-term enrichment using Blast2go, with significantly over-represented terms (Fisher's exact test, FDR<0.05) summarised in Figures S2, S3, S4, S5, S6, S7, S8, S9 and S10. This analysis also revealed significantly under-represented terms, highlighting which process and functions were less likely to contribute specific phenotypic traits of *D. bruxellensis*.

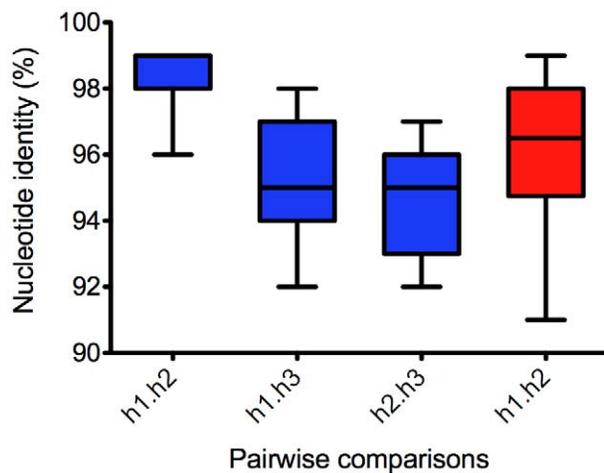


Figure 3. Pairwise comparison of ORF haplotypes. Manually extracted haplotypes were aligned, and pairwise nucleotide identity calculated. For ORFs represented by three haplotypes, the pair with highest nucleotide identity were assigned to haplotype 1 (h1) and haplotype 2 (h2).
doi:10.1371/journal.pone.0033840.g003

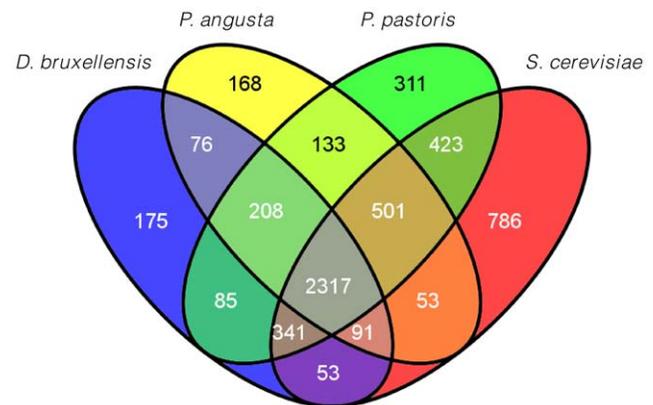


Figure 4. Venn diagram of OrthoMCL cluster distribution across *D. bruxellensis*, *P. angusta*, *P. pastoris*, and *S. cerevisiae*.
doi:10.1371/journal.pone.0033840.g004

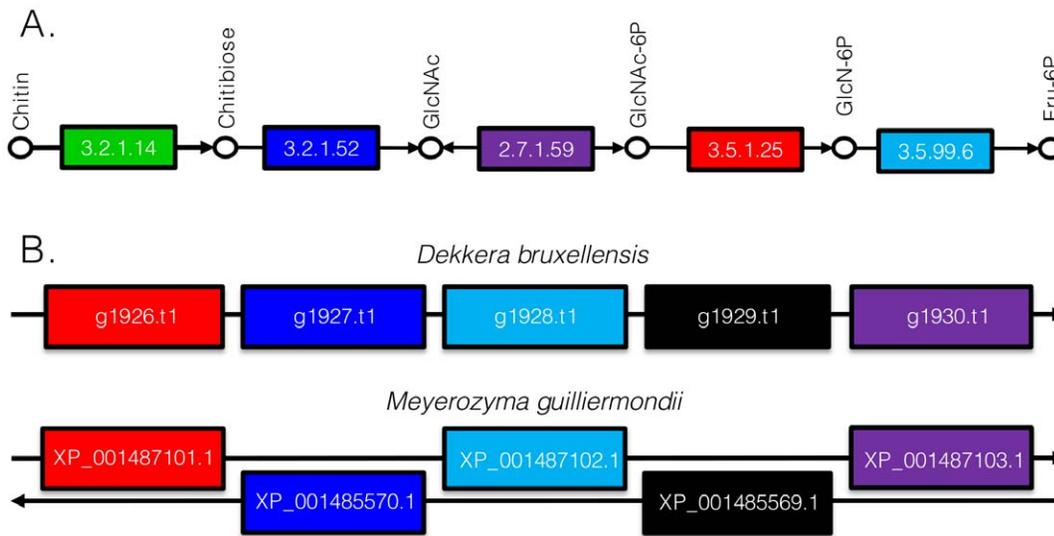


Figure 5. Metabolic capability of *D. bruxellensis* revealed through OrthoMCL analysis. Chitin/N-acetylglucosamine assimilation pathway (A) and arrangement of gene cluster in *D. bruxellensis* AWRI1499 and *M. guilliermondii* (B). Pathway redrawn from KEGG map (ko00520). ORF numbers for *D. bruxellensis* proteins and accession numbers for *M. guilliermondii* orthologs mapped to chitin/N-acetylglucosamine assimilation pathway, indicated by shared box colour. Chitinase (3.2.1.14) not located in cluster, g1929.t1 & XP_001485569 members of MFS transporter superfamily. doi:10.1371/journal.pone.0033840.g005

Proteins corresponding to OrthoMCL clusters expanded in *D. bruxellensis* were highly enriched in GO terms corresponding to the cell membrane. OrthoMCL cluster OG5_126579 was quantitatively the most expanded in *D. bruxellensis*. This cluster, to which *S. cerevisiae* ORFs *FIG2*, *FLO1*, *FLO5*, *FLO9*, *HKR1*, *HPF1*, *MSB2*, *MUC1*, *PRM7*, *YIL169C*, and *YMR317W*, were assigned, encompasses divergent plasma membrane and cell wall proteins involved in cell wall budding, adhesion, and pseudohyphal growth. *D. bruxellensis* has been shown to form biofilms [24] and pseudohyphae [25] - traits that would be advantageous for survival in wine stored in oak barrels [10] where they would provide the means of adhering to the internal wall of the barrel and resistance to removal by high pressure cleaning. Membrane associated transporters were also significantly expanded in *D. bruxellensis*. Amino acid permeases were expanded compared to all three species, while the *D. bruxellensis/Pichia* lineage expansion was enriched in transmembrane drug transporters, and *D. bruxellensis/S. cerevisiae* expanded clusters were enriched in nucleobase transporters. Survey sequencing of *D. bruxellensis* strain CBS 2499 also revealed an enrichment of transporters, consistent with adaptation to relatively low nutrient environments like wine [14].

Despite exposure to the common wine preservative, sulfite, *D. bruxellensis* AWRI1499 has only one ORF (g80.t1) that encodes a protein with homology to the *S. cerevisiae* sulfite efflux transporter, Ssu1p. This protein confers sulfite tolerance [26] and has been the subject of positive selection in vineyard *S. cerevisiae* populations exposed to sulfite [27]. In *S. cerevisiae*, *SSU1* is duplicated, and strains with high levels of sulfite tolerance harbor one or more *SSU1-R* (resistant) alleles [28] that are highly expressed. Furthermore, a sulfite inducible *SSU1-R* allele was recently described for the wine yeast *S. cerevisiae* 71B [29]. Genomic and transcriptomic comparison of multiple *D. bruxellensis* strains will be required to determine whether molecular mechanisms conferring sulfite tolerance are similar for the two species.

GO terms corresponding to oxidoreductase catalytic activity were also a feature in OrthoMCL clusters expanded in all comparisons. This may reflect a strategy evolved to enable survival in anaerobic conditions where the species has impaired capacity to

regenerate NAD(P)⁺. *D. bruxellensis* contains eight oxidoreductases corresponding to the OrthoMCL cluster of alcohol dehydrogenase proteins (Figure 6A), and five corresponding to aldehyde dehydrogenase (Figure 6B), while also showing expansion in other, less specific, oxidoreductase clusters. Oxidation of acetaldehyde to acetate, and possible imbalance in subsequent acetyl-CoA and/or succinate formation, has been linked to overproduction of acetate under aerobic conditions, and the Custers effect (inhibition of fermentation under anaerobic conditions) [30]. In this context it is interesting to note that the closest protein sequence match for the *D. bruxellensis* Ald4p ortholog is an acetaldehyde dehydrogenase recently cloned and characterised from *Issatchenkia terricola*, which was found to be significantly more active than *S. cerevisiae* aldehyde dehydrogenase enzymes in metabolising acetaldehyde [31].

In *S. cerevisiae*, alcohol and aldehyde dehydrogenases, along with other oxidoreductase enzymes, contribute to the formation of aroma compounds through the Ehrlich pathway [32]. The balance between oxidation and reduction determines, for example, formation of isoamyl-alcohol and its well known ester isoamyl-acetate, or alternatively isovaleric acid. *D. bruxellensis* has been reported to produce the latter compound, which has a 'rancid' aroma thought to contribute to the sensory impact of *D. bruxellensis* on wine [33]. In this context it is worth noting that while hydrolase OrthoMCL clusters were not significantly expanded according to the GO-term enrichment analysis, three *D. bruxellensis* proteins were found that each have homology to isoamyl-acetate hydrolysis enzymes (Figure 7). Two homologs present as a tandem repeat (g3110.t1, g3111.t1) were most similar to the *S. cerevisiae* Iah1p enzyme which, in conjunction with Atf1p, determines concentrations of isoamyl alcohol and isoamyl acetate that are produced during fermentation [34].

A key trait of *D. bruxellensis* is the capacity to take up hydroxycinnamic acids and convert them to their respective ethylphenols [10]. This metabolic pathway requires an enzyme with phenolic acid decarboxylase (PAD) activity, an activity found across many fungal and bacterial species, and an enzyme with vinylphenol reductase (VPR) activity. Aside from *D. bruxellensis*, the

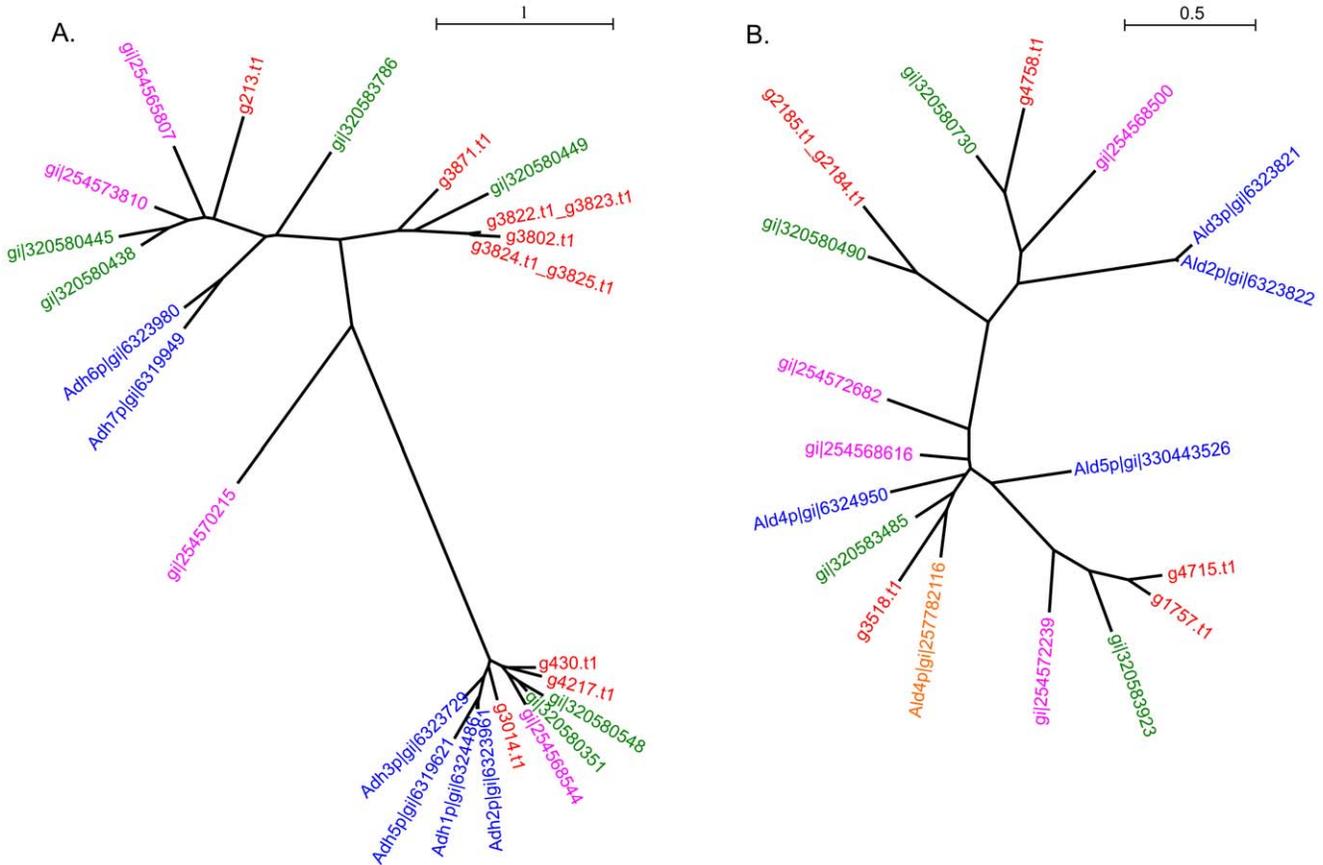


Figure 6. OrthoMCL oxidoreductase clusters expanded in *D. bruxellensis*. Alcohol dehydrogenase (A), and aldehyde dehydrogenase (B). Unrooted maximum likelihood trees generated from amino acid alignments of proteins assigned to the same OrthoMCL cluster. Red = *D. bruxellensis*, blue = *S. cerevisiae*, green = *P. angusta*, pink = *P. pastoris*, orange = *Issatchenkia terricola*. doi:10.1371/journal.pone.0033840.g006

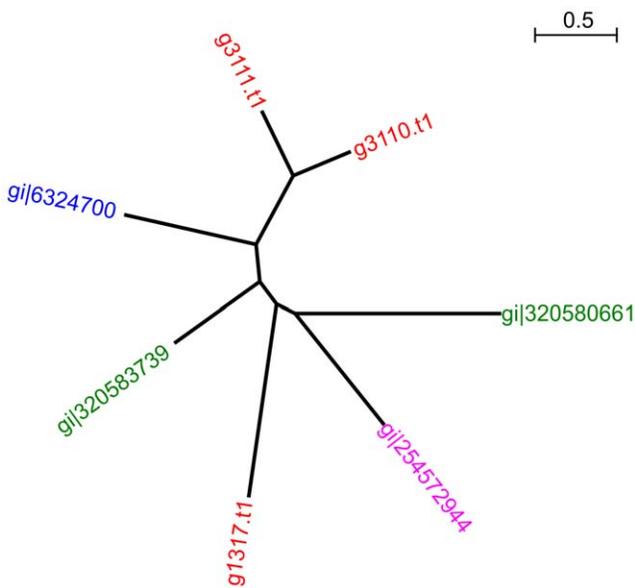


Figure 7. OrthoMCL cluster expanded in *D. bruxellensis*, corresponding to isoamyl-acetate hydrolase. Unrooted maximum likelihood trees generated from amino acid alignments of proteins from each species assigned to the same OrthoMCL cluster. Red = *D. bruxellensis*, blue = *S. cerevisiae*, green = *P. angusta*, pink = *P. pastoris*. doi:10.1371/journal.pone.0033840.g007

only other wine-associated yeast species demonstrated to convert vinylphenols to ethylphenols through this enzymatic activity is *M. guilliermondii* [35].

Tchobanov et al. [36] partially purified a 26 kDa protein that exhibited VPR activity, and assembled a putative VPR ORF from CBS 2499 [14] contigs against peptide fragment sequences. Blastn of DbVPR against the AWRI1499 ORFs revealed a close match (99% nucleotide identity) with g4418.t1. This protein was, however, annotated by Blast2go as a carboxypeptidase-y inhibitor, of the phosphatidylethanolamine-binding protein (PEBP) family, sharing 58% amino acid identity with orthologous protein from *P. angusta*. Proteins of this family do not have NAD(H) or NADP(H) binding domains, thus it seems questionable that DbVPR indeed performs the VPR function. Godoy et al. [37] also reported purification of a putative VPR enzyme from *D. bruxellensis*, which exhibited very different kinetic properties. Finding the gene that encodes this critical enzyme in *D. bruxellensis* may be possible by functional analysis of the oxidoreductase OrthoMLC clusters expanded in *D. bruxellensis*.

Characterised proteins with PAD activity belong to two InterProScan families; phenylacrylic acid decarboxylase (IPR004507) which includes *S. cerevisiae* Pad1p, and the bacterial phenolic acid decarboxylases (IPR008729). No homolog of the *S. cerevisiae* protein Pad1p was found in the partial sequence of *D. bruxellensis* CBS 2499 [14], nor did we find one in the genomic assembly of AWRI1499. We therefore screened the *D. bruxellensis* genome sequence for potential PAD-encoding genes using

characterised bacterial proteins from *Bacillus pumilus*, *Bacillus subtilis*, *Pediococcus pentosaceus* and *Lactobacillus plantarum*. No significant hits were found amongst the *D. bruxellensis* ORFs, however a significant tblastn hit ($E < 1e-30$) was found in contig 3340 of scaffold 100. A hypothetical protein, DbPad, was generated by fusing two frameshifted (due to assembly sequence errors) translations, and aligned against fungal proteins with homology to DbPad or *S. cerevisiae* Pad1p. The resultant maximum likelihood tree (Figure 8) shows that several ascomycete and basidiomycete proteins share homology with the putative DbPad, which is more similar to bacterial phenolic acid decarboxylase proteins than the *S. cerevisiae* Pad1 protein. The tree also highlights that several filamentous fungi have both Pad1p- and DbPad-like proteins.

PAD activity in *D. anomala* was attributed to a protein with homology to *S. cerevisiae* Pst2p, a member of the flavodoxin family [38]. Three *D. bruxellensis* AWRI1499 ORFs encode Pst2 homologs, exhibiting 99% (g3584.t1), 67% (g3585.t1) and 57% (g797.t1) amino acid similarity to the *D. anomala* Pst2 protein. These proteins do not share homology with the phenylacrylic acid decarboxylase, or phenolic acid decarboxylase families. Nonetheless, it is possible that through duplication of Pst2 in *D. bruxellensis* AWRI1499 and subsequent functional divergence, PAD activity can be attributed to one of the three proteins. On the other hand, it is interesting to note that *M. guilliermondii* was the only budding yeast found to contain a DbPad homolog (Figure 8). An enzyme with phenolic acid decarboxylase activity was recently purified from *M. guilliermondii* [39], and found to have a molecular mass of 20 kDa, the predicted size of the DbPad protein. Furthermore, this protein exhibited a substrate specificity towards hydroxycinnamic acids favouring *p*-coumaric acid and ferulic acid over caffeic acid. Caffeic acid is typically more abundant than *p*-coumaric acid in wine, particularly when esterified with tartaric acid [40–42], whereas wine affected by *D. bruxellensis* exhibits higher concentrations of 4-ethylphenol (derived from *p*-coumaric acid) than 4-ethylcatechol [43]. We can, therefore, speculate that the dominant sensory characteristics attributed to *D. bruxellensis* spoilage of wine are a consequence of DbPad substrate specificity. Availability of the AWRI1499 genomic assembly and annotation will facilitate functional characterisation of the DbPAD gene, and others that may confer industrially relevant phenotypic traits.

Conclusion

This study sheds light on an industrially important microorganism which, because of its intractable nature, has been poorly understood. Analysis of gene content points to an organism that is metabolically well equipped to survive in challenging environments such as wine. The genomic assembly described in the current study reveals a triploid genome, consistent with speciation through inter-specific hybridisation and an asexual lifestyle. Availability of the genomic assembly and annotation will facilitate advancement in our knowledge of *D. bruxellensis* biology, both from an evolutionary perspective and in terms of its role in fermentation processes.

Materials and Methods

Yeast strain, nucleic acid preparation, and sequencing

D. bruxellensis strain AWRI1499 was obtained from The Australian Wine Research Institute Microorganisms Culture Collection. Genomic DNA was prepared using a standard zymolyase and phenol-chloroform extraction from cultures grown under standard conditions. RNA was extracted from cultures grown under standard conditions and incubated for 2 h in the presence of 2 mM ferulic acid to induce a stress response [44],

using Trizol (Invitrogen), RNase-free DNase (Qiagen) treatment, and purified using the Purelink RNA Mini Kit (Invitrogen). Shotgun and paired-end library construction and sequencing of genomic DNA was performed by 454 Life Sciences, A Roche Company (Branford, CT) using GS-FLX Titanium series chemistry and their own standard protocols. Of 1,859,751 total reads generated, 1,227,460 were paired-end. Average read lengths were 345 bp for the paired-end library and 369 bp for the shotgun library. RNAseq was performed at The Ramaciotti Centre for Gene Function Analysis (Sydney, NSW) using 2×100 bp paired end libraries on the Illumina HiSeq2000.

De-novo genome and transcriptome assembly

MIRA (v3rc2) [45] was used to assemble shotgun and paired-end reads. The resultant assembly file, comprising 84% of sequencing reads, was imported into Lasergene Seqman (v8) for manual finishing. All vs all Blastn was performed for ‘small’ contigs and ‘large’ contigs (arbitrary size cutoff), and a custom python script developed that sequentially unlocked reciprocal best-hit contigs ($E\text{-value} < 1e-30$) in Seqman, and reassembled the ‘small’ contig into the matching ‘large’ contig, before locking all contigs. ‘Small’ contigs remaining after this process were reverse-complemented and reassembled against their reciprocal blastn best-hit large contig. Through this process the number of contigs was reduced to ~6,000. Default Seqman parameters were used during manual assembly (match size 12, min match % 80, min seq length 100, max added gaps per kb 70, gap penalty 0.00, gap length penalty 0.70). Further manual assembly was guided by paired end information that inferred either an end-to-end join, extending length of contig/scaffold, or an internal assembly of a small contig into a larger contig. Blastn alignments were performed to determine appropriate course of action (align, assemble, or reverse-complement then assemble). Each reassembled contig was manually examined for consistency of paired-end data. Consensus sequences were exported with SNP detection at 50% of reads, and reimported into Seqman for subsequent alignment of INDEL-containing contigs. RNAseq reads were pooled and assembled using the Trinity pipeline [46], then transcript contig sequences imported into Seqman and assembled against the reference genomic assembly, to aid homopolymer detection and correction in putative open reading frames. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/Genbank under the accession AHIQ00000000. The version described in this paper is the first version, AHIQ01000000.

Regions of copy number variation were determined by calculating the per-base sequencing coverage across each sequencing contig with median smoothing (1001 bp window, 100 by step size). The ratio between coverage at each genomic location and overall median genomic coverage was calculated to determine the level of over- or under-representation for each location. Large scale aneuploidies were detected by screening for regions where median ratio for a contiguous stretch of at least 101 individual segments differed from the overall genomic median by either 1.25- or 1.5-fold.

Open reading frame prediction and annotation

Open reading frame prediction was performed using Augustus v2.5.5 [18], incorporating open reading frame hints generated by mapping of RNAseq data to the MIRA assembly using BLAT. *S. cerevisiae* and *P. pastoris* gene models built in Augustus were compared for prediction of *D. bruxellensis* ORFs, with the *S. cerevisiae* based model resulting in a higher number of predicted ORFs and therefore preferred. ORFs were visualised in IGV

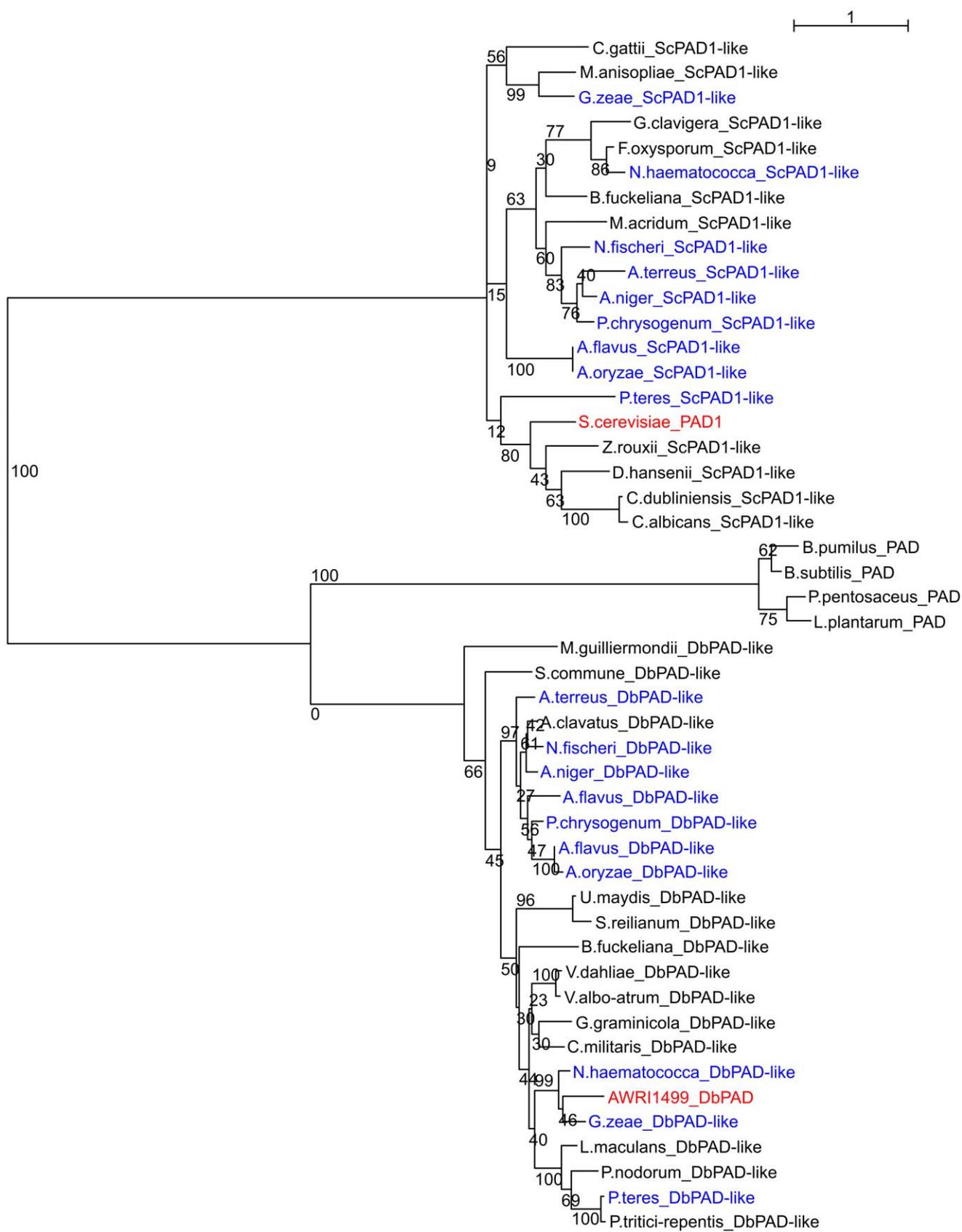


Figure 8. Phylogeny of fungal phenolic acid decarboxylase enzymes. Maximum likelihood tree generated from amino acid alignment of all fungal proteins with homology to *S. cerevisiae* Pad1p (ScPAD1) or putative *D. bruxellensis* Padp (DbPAD). Species with homologs of both proteins indicated in blue. Bootstrap values (100 randomisations) for all nodes shown.
doi:10.1371/journal.pone.0033840.g008

v2.03 [47] with mapped RNAseq reads in order to identify and correct erroneous frameshifts due to 454 homopolymers (editing performed in Seqman). ORFs were then annotated using Blast2go v2.5 [19]. A custom perl script was used to evaluate codon usage and proportion of non-synonymous SNPs in ORFs.

Phylogenetic analysis

Twenty species were identified in Blast2go as most frequent ‘top-hit’ in blastp searches conducted using the *D. bruxellensis* predicted proteins. The proteome for each species was used in pairwise blastp against *D. bruxellensis*, and reciprocal top-hits identified as putative orthologs. A table of putative orthologs across all 21 species is available in supplementary data (Table S3). Proteins present across all species (542) were separately aligned using the MUSCLE algorithm in Seaview [48], concatenated, then submitted to PhyML [49] for construction of a maximum likelihood tree, with 1000 randomisations. Phylogenetic analysis of specific proteins were also performed using this approach.

Comparative ortholog cluster analysis

The predicted proteomes of *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae* were submitted to the OrthoMCL web server [50] for assignment to orthologous clusters. Overlapping (and unique) clusters were identified using Venny [51]. Lists of *D. bruxellensis* ORFs from OrthoMCL clusters found to be unique or expanded in the four-way comparison were manually curated for instances where sequential open reading frames in AWRI1499 were assigned to the same cluster. If these ORFs represented partial hits to different parts of the same protein in blast2go, or the sequential ORFs were within a region encompassed by tblastn of the relevant blast2go top-hit against the scaffold assembly, the sequential ORFs were assumed to comprise a single gene. Curated ORF lists were then tested for GO-term enrichment using Fisher’s exact test in Blast2go.

Supporting Information

Figure S1 Distribution of Blastp top-hits against non-redundant database performed by Blast2go for all predicted *D. bruxellensis* AWRI1499 proteins. (TIF)

Figure S2 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Biological process GO-terms enriched in comparison to all three *spp.* (PDF)

Figure S3 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Cell component GO-terms enriched in comparison to all three *spp.* (PDF)

Figure S4 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Molecular function GO-terms enriched in comparison to all three *spp.* (PDF)

Figure S5 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P.*

***angusta*, and *S. cerevisiae*.** Biological process GO-terms enriched in comparison to *P. pastoris* and *P. angusta*, but not *S. cerevisiae*. (PDF)

Figure S6 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Cellular component GO-terms enriched in comparison to *P. pastoris* and *P. angusta*, but not *S. cerevisiae*. (PDF)

Figure S7 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Molecular function GO-terms enriched in comparison to *P. pastoris* and *P. angusta*, but not *S. cerevisiae*. (PDF)

Figure S8 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Biological process GO-terms enriched in comparison to *S. cerevisiae*, but not *P. pastoris* and *P. angusta*. (PDF)

Figure S9 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Cellular component GO-terms enriched in comparison to *S. cerevisiae*, but not *P. pastoris* and *P. angusta*. (PDF)

Figure S10 GO-term enrichment analysis for OrthoMCL clusters common to *D. bruxellensis*, *P. pastoris*, *P. angusta*, and *S. cerevisiae*. Molecular function GO-terms enriched in comparison to *S. cerevisiae*, but not *P. pastoris* and *P. angusta*. (PDF)

Table S1 Contig statistics. Average sequence coverage and length of contigs ordered by position within scaffolds. (XLS)

Table S2 *D. bruxellensis* open reading frame ortholog group assignments. OrthoMCL assignments and sequence ID of best hit for all predicted protein coding open reading frames. (XLS)

Table S3 Putative orthologs of *D. bruxellensis* proteins across 20 fungal species. The proteomes of species frequently assigned as top blastp hit for *D. bruxellensis* proteins in Blast2Go were used in pairwise blastp against the *D. bruxellensis* proteome, and reciprocal top-hits identified as putative orthologs. (XLS)

Acknowledgments

The authors would like to thank Toni Cordente, Robyn Kievit, and Sylvester Holt for technical support.

Author Contributions

Conceived and designed the experiments: CDC ARB PJC ISP. Performed the experiments: CDC ARB. Analyzed the data: CDC ARB. Wrote the paper: CDC ARB PJC ISP.

References

- Teoh A, Heard G, Cox J (2004) Yeast ecology of Kombucha fermentation. *Int J Food Microbiol* 95(2): 119–126.
- Meroth CB, Hammes WP, Hertel C (2003) Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 69: 7453–7461.
- Passoth V, Blomqvist J, Schnürer J (2007) *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* 73: 4354–4356.
- Galafassi S, Merico A, Piza F, Hellborg L, Molinari F, et al. (2010) *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. *J Ind Microbiol Biotechnol* 38(8): 1079–1088.
- Blomqvist J, South E, Tiukova L, Momeni MH, Hansson H, et al. (2011) Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Dekkera bruxellensis*. *Lett Appl Microbiol* 53: 73–78.
- Morrissey WF, Davenport B, Querol A, Dobson ADW (2004) The role of indigenous yeasts in traditional Irish cider fermentations. *J Appl Microbiol* 97: 647–655.
- Curtin CD, Bellon JR, Henschke PA, Godden PW, de Barros Lopes MA (2007) Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Res* 7: 471–481.
- Rozpedowska E, Hellborg L, Ishchuk OP, Orhan F, Galafassi S, et al. (2011) Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. *Nat Commun* 2: 302. doi:10.1038/ncomms1305.
- Fugelsang K (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (*Vitis vinifera* L.) wines. *Am J Enol Viticult* 54(4): 294–300.
- Chatonnet P, Dubourdie D, Boidron J, Pons M (1992) The origin of ethylphenols in wines. *J Sci Food Agric* 60(2): 165–178.
- Lathey KA, Bramley BR, Francis IL (2010) Consumer acceptability, sensory properties and expert quality judgements of Australian Cabernet Sauvignon and Shiraz wines. *Aust J Grape Wine Res* 16: 189–202.
- Loureiro V, Malfaito-Ferreira M (2003) Spoilage yeasts in the wine industry. *Int J Food Microbiol* 86: 23–50.
- Vanbeneden N, Gils F, Delvaux F, Delvaux FR (2008) Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. *Food Chem* 107(1): 221–230.
- Woolfit M, Rozpedowska E, Piškur J, Wolfe KH (2007) Genome Survey Sequencing of the Wine Spoilage Yeast *Dekkera (Brettanomyces) bruxellensis*. *Eukaryot Cell* 6: 721–733.
- Hellborg L, Piškur J (2009) Complex Nature of the Genome in a Wine Spoilage Yeast, *Dekkera bruxellensis*. *Eukaryot Cell* 8: 1739–1749.
- Poláčková E, Poláčková S, Piškur J, Sulo P (2010) Mitochondrial genome from the facultative anaerobe and petite-positive yeast *Dekkera bruxellensis* contains the NADH dehydrogenase subunit genes. *FEMS Yeast Res* 10: 545–557.
- Harris V, Jiranek V, Ford CM, Grbin PR (2009) Inhibitory effect of hydroxycinnamic acids on *Dekkera* spp. *Appl Microbiol Biotechnol* 86: 721–729.
- Stanke M, Steinkamp R, Waack S, Morgenstern B (2004) AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Res* 32: W309–12. doi:10.1093/nar/gkh379.
- Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21: 3674–3676.
- Parra G, Bradnam K, Ning Z, Keane T, Korfi I (2009) Assessing the gene space in draft genomes. *Nucleic Acids Res* 37: 289–297.
- Nakao Y, Kanamori T, Itoh T, Kodama Y, Rainieri S, et al. (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Res* 16: 115–129.
- Antunovic Z, Nguyen HV, Gaillardin C, Sipiczki M (2005) Gradual genome stabilisation by progressive reduction of the *Saccharomyces uvarum* genome in an interspecific hybrid with *Saccharomyces cerevisiae*. *FEMS Yeast Res* 5: 1141–1150.
- Delneri D, Colson I, Grammenoudi S, Roberts IN, Louis EJ, et al. (2003) Engineering evolution to study speciation in yeasts. *Nature* 422: 68–72.
- Joseph CM, Kumar G, Su E, Bisson LF (2007) Adhesion and biofilm production by wine isolates of *Brettanomyces bruxellensis*. *Am J Enol Viticult* 58(3): 373–378.
- Van der Walt JP (1964) *Dekkera*, New Genus of Saccharomycetaceae. *Antonie Van Leeuwenhoek* 30: 273–280.
- Avram D, Bakalinsky AT (1997) *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*. *J Bacteriol* 179(18): 5971–5974.
- Aa E, Townsend JP, Adams RI, Nielsen KM, Taylor JW (2006) Population structure and gene evolution in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 6: 702–715.
- Goto-Yamamoto N, Kitano K, Shiki K, Yoshida Y, Suzuki T, et al. (1998) *SSU1-R*, a sulfite resistance gene of wine yeast, is an allele of *SSU1* with a different upstream sequence. *J Ferment Bioengineer* 86: 427–433.
- Nardi T, Corich V, Giacomini A, Blondin B (2010) A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. *Microbiol* 156: 1686–1696.
- Carracosa J, Viguera M, Núñez de Castro I, Scheffers WA (1981) Metabolism of Acetaldehyde and Custers Effect in the Yeast *Brettanomyces abstinentis*. *Antonie van Leeuwenhoek* 47: 209–215.
- Yao Z, Zhang C, Lu F, Bie X, Lu Z (2011) Gene cloning, expression, and characterization of a novel acetaldehyde dehydrogenase from *Issatchenkia terricola* strain XJ-2. *Appl Microbiol Biotechnol* doi:10.1007/s00253-011-3541-7.
- Hazelwood LA, Daran JM, van Maris AJA, Pronk JT, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* 74: 2259–2266.
- Romano A, Perello MC, de Revel G, Lonvaud-Funel A (2008) Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *J Appl Microbiol* 104: 1577–1585.
- Lilly M, Bauer FF, Lambrechts MG, Swiegers JH, Cozzolino D, et al. (2006) The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast* 23: 641–659.
- Barata A, Nobre A, Correia P, Malfaito-Ferreira M, Loureiro V (2006) Growth and 4-ethylphenol production by the yeast *Pichia guilliermondii* in grape juices. *Am J Enol Viticult* 57: 133–138.
- Tchobanov I, Gal L, Guilloux-Benatier M, Remize F, Nardi T, et al. (2008) Partial vinylphenol reductase purification and characterization from *Brettanomyces bruxellensis*. *FEMS Microbiol Lett* 284: 213–217.
- Godoy L, Martínez C, Carrasco N, Ganga MA (2008) Purification and characterization of a *p*-coumarate decarboxylase and a vinylphenol reductase from *Brettanomyces bruxellensis*. *Int J Food Microbiol* 127: 6–11.
- Harris V, Ford CM, Jiranek V, Grbin PR (2008) Survey of enzyme activity responsible for phenolic off-flavour production by *Dekkera* and *Brettanomyces* yeast. *Appl Microbiol Biotechnol* 81: 1117–1127.
- Huang H-K, Tokashiki M, Maeno S, Onaga S, Taira T, et al. (2011) Purification and properties of phenolic acid decarboxylase from *Candida guilliermondii*. *J Ind Microbiol Biotechnol*. doi:10.1007/s10295-011-0998-4.
- Nagel C, Baranowski J, Wulf LW, Powers JR (1979) The hydroxycinnamic acid tartaric acid ester content of musts and grape varieties grown in the Pacific Northwest. *Am J Enol Viticult* 30(3): 198–201.
- Betés-Saura C, Andrés-Lacueva C, Lamuela-Raventós RM (1996) Phenolics in white free run juices and wines from Penedès by High-Performance Liquid Chromatography: changes during vinification. *J Agric Food Chem* 44: 3040–3046.
- Boido E, Garca-Marino M, Dellacassa E, Carrau F, Rivas-Gonzalo JC, et al. (2011) Characterisation and evolution of grape polyphenol profiles of *Vitis vinifera* L. cv. Tannat during ripening and vinification. *Aust J Grape Wine Res* 17(3): 383–393.
- Larcher R, Nicolini G, Bertoldi D, Nardin T (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography–coulometric electrochemical array detection. *Anal Chim Acta* 609: 235–240.
- Harris V, Ford CM, Jiranek V, Grbin PR (2008) *Dekkera* and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media. *Appl Microbiol Biotechnol* 78: 997–1006.
- Chevreaux B (2005) MIRA: An automated genome and EST assembler. Thesis. Ruprecht-Karls University, Duisburg.
- Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29: 644–652.
- Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, et al. (2011) Integrative genomics viewer. *Nat Biotechnol* 29: 24–26.
- Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27: 221–224.
- Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online—a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res* 33(suppl 2): W557–W559. doi: 10.1093/nar/gki352.
- Li L, Stoeckert CJ, Roos DS (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res* 13: 2178–2189.
- Oliveros J (2007) VENNY. An interactive tool for comparing lists with Venn diagrams. <http://bioinfop.cnb.csic.es/tools/venny/index.html>.

**P L o S One**[◀ BACK TO RESULTS](#)[Click for Open Access](#)**JCR** Web

Click highlighted text for a new search on that item.

| | |
|----------------------------|---|
| ISSN: | 1932-6203 |
| Title: | P L o S One Additional Title Information |
| Publishing Body: | Public Library of Science |
| Country: | United States |
| Status: | Active |
| Start Year: | 2006 |
| Frequency: | Irregular |
| Document Type: | Journal: Academic/Scholarly |
| Refereed: | Yes |
| Abstracted/Indexed: | Yes |
| Media: | Online - full text |
| Language: | Text in English |
| Price: | Free |
| Subject: | SCIENCES: COMPREHENSIVE WORKS MEDICAL SCIENCES |
| Dewey #: | 500, 610 |
| LC#: | Q179.9 |
| Editor(s): | Damian Pattinson |
| Publisher(s): | Peter Binfield |
| URL: | http://www.plosone.org/home.action |
| Description: | Covers primary research from all disciplines within science and medicine. |

ADDITIONAL TITLE INFORMATION

Acronym Description: Public Library of Science
Alternate Title: Variant format: PLoS ONE

[▲ Back to Top](#)

| | |
|--|---|
| Add this item to: (select a list) <input type="button" value="+ ADD"/> | Request this title: I'd like to request this title. <input type="button" value="GO"/> |
| | Corrections: Submit corrections to Ulrich's about this title. <input type="button" value="GO"/> |
| | Publisher of this title? If yes, click GO! to contact Ulrich's about updating your title listings in the Ulrich's database. <input type="button" value="GO"/> |

[Print](#) • [Download](#) • [E-mail](#)[▲ Back to Top](#)