Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*

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# ABSTRACT

Tardigrada, a phylum of meiofaunal organisms, have been at the center of discussions of the evolution of Metazoa, the biology of survival in extreme environments, and the role of horizontal gene transfer in animal evolution. Tardigrada are placed as sisters to Arthropoda and Onychophora (velvet worms) in the superphylum Panarthropoda by morphological analyses, but many molecular phylogenies fail to recover this relationship. This tension between molecular and morphological understanding may be very revealing of the mode and patterns of evolution of major groups. Limno-terrestrial tardigrades display extreme cryptobiotic abilities, including anhydrobiosis and cryobiosis, as do bdelloid rotifers, nematodes and other animals of the water film. These extremophile behaviors challenge understanding of normal, aqueous physiology: how does a multicellular organism avoid lethal cellular collapse in the absence of liquid water? Meiofaunal species have been reported to have elevated levels of HGT events, but how important this is in evolution, and particularly in the evolution of extremophile physiology, is unclear. To address these questions, we resequenced and reassembled the genome of *Hypsibius dujardini*, a limno-terrestrial tardigrade that can undergo anhydrobiosis only after extensive pre-exposure to drying conditions, and compared it to the genome of *Ramazzottius varieornatus*, a related species with tolerance to rapid desiccation. The two species had contrasting gene expression responses to anhydrobiosis, with major transcriptional change in *H. dujardini* but limited regulation in *R. varieornatus*. We identified few horizontally transferred genes, but some of these were shown to be involved in entry into anhydrobiosis. Whole-genome molecular phylogenies supported a Tardigrada+Nematoda relationship over Tardigrada+Arthropoda, but rare genomic changes tended to support Tardigrada+Arthropoda.

# INTRODUCTION

The superphylum Ecdysozoa emerged in the Precambrian, and ecdysozoans not only dominated the early Cambrian explosion but are also dominant (in terms of species, individuals and biomass) today. The relationships of the eight phyla within Ecdysozoa remain contentious, with morphological assessments, developmental analyses and molecular phylogenetics yielding conflicting signals [1-3]. It has generally been accepted that Arthropoda, Onychophora (the velvet worms) and Tardigrada (the water bears or moss piglets) form a monophylum, Panarthropoda [2], and that Nematoda (roundworms) are closely allied to Nematomorpha (horsehair worms), and distinct from Panarthropoda. However, molecular phylogenies have frequently placed representatives of Tardigrada as sisters to Nematoda [1, 3], invalidating Panarthropoda and challenging models of the evolution of complex morphological traits such as segmentation, serially repeated lateral appendages, the triradiate pharynx and a tripartite central nervous system [4, 5].

The key taxon in these disagreements is phylum Tardigrada. Nearly 1,200 species of tardigrades have been described [6]. All are members of the meiofauna - small animals that live in the water film and in interstices between sediment grains [6]. There are marine, freshwater and terrestrial species. Many species of terrestrial tardigrades are cryptobiotic: they have the ability to survive extreme environmental challenges by entering a dormant state [7]. Common to these resistances is an ability to lose or exclude the bulk of body water, and anhydrobiotic tardigrades have been shown to have tolerance to high and low temperatures (including freezing), organic solvents, X- and gamma-rays, high pressure and the vacuum of space [8-15]. The physiology of anhydrobiosis in tardigrades has been explored extensively, but little is currently known about its molecular bases [16, 17]. Many other animals have cryptobiotic abilities, including some nematodes and arthropods [18], and comparison of the mechanisms in different independent acquisitions of this trait will reveal underlying common mechanisms.

Central to the development of tractable experimental models for cryptobiosis is the generation of high-quality genomic resources. Genome assemblies of two tardigrades, *Hypsibius dujardini* [19-21] and *Ramazzottius varieornatus* [22], both in the family Hypsibiidae, have been published. *H. dujardini* is a limno-terrestrial tardigrade which is easy to culture [23], while *R. varieornatus* is a terrestrial tardigrade, and highly tolerant of environmental extremes [24]. An experimental toolkit for *H. dujardini*, including RNAi and *in situ* hybridization is being developed [25]. *H. dujardini* is poorly cryptobiotic compared to *R. varieornatus*. *H. dujardini* requires 48 h of preconditioning at 85% relative humidity (RH) and further 24 h in 30% RH [23] to enter cryptobiosis with high survival, while *R. varieornatus* can form a tun (the cryptobiotic form) within 30 min at 30% RH [26].

Several anhydrobiosis-related genes have been identified in Tardigrada. Catalases, superoxide dismutases (SOD), and glutathione reductases may protect against oxidative stress [27], and chaperones, such as heat shock protein 70 (HSP70) [28-30], may act to protect proteins from the denaturing effects of water loss [16, 31, 32]. Additionally, several tardigrade-specific gene families have been implicated in anhydrobiosis, based on their expression patterns. Cytosolic abundant heat soluble (CAHS), secretory abundant heat soluble (SAHS), late embryogenesis abundant protein mitochondrial (RvLEAM), mitochondrial abundant heat soluble protein (MAHS), and damage suppressor (Dsup) gene families have been implicated in *R. varieornatus* extremo-tolerance [22, 33, 34]. These gene families were named by their subcellular location or function, and expression of MAHS and Dsup in human tissue culture cell lines resulted in elevated levels of tolerance against osmotic stress and X-ray irradiation (~4 Gy). Surprisingly, analyses of the *R. varieornatus* genome showed extensive gene loss in the peroxisome pathway and in stress signaling pathways, suggesting that this species is compromised in terms of reactive oxygen resistance and repair of cellular damage [22]. While loss of these pathways would be lethal for a normal organism, loss of these resistance pathways may be associated with anhydrobiosis.

Desiccation in some taxa induces the production of anhydroprotectants, small molecules that likely replace cellular water to stabilize cellular machinery. Trehalose, a disaccharide shown to contribute to anhydrobiosis in midges [35, 36], nematodes [37] and artemia [38], is not present in the tardigrade *Milnesium tardigradum* [31]. Coupled with the ability of *R. varieornatus* to enter anhydrobiosis rapidly (*i.e.* without the need for extensive preparatory biosynthesis), this suggests that tardigrade anhydrobiosis does not rely on induced synthesis of protectants. Entry into anhydrobiosis in *H. dujardini* does require active transcription during preconditioning, suggesting the activation of a genetic program to regulate physiology. Inhibition of PP1/2A, an positive regulator of the FOXO transcription factor which induces anti-oxidative stress pathways, led to high lethality in *H. dujardini* during anhydrobiosis induction [23]. As *R. varieornatus* does not require preconditioning, systems critical to anhydrobiosis in *R. varieornatus* are likely to be constitutively expressed.

*H. dujardini* and *R. varieornatus* are relatively closely related (both are members of Hypsibiidae), and both have available genome sequences. The *R. varieornatus* genome has high contiguity and scores highly in all metrics of gene completeness [22]. For *H. dujardini*, three assemblies have been published. One has low contiguity (N50 length of 17 kb) and contains a high proportion of contaminating non-tardigrade sequence, including ~40 Mb of bacterial sequence, and spans 212 Mb [19]. The other two assemblies, both at ~130 Mb [20, 21], eliminated most contamination, but contained uncollapsed haploid segments because of unrecognized heterozygosity (estimated to be around 30~60%, or ~30 Mb) from k-mer distributions. The initial, low quality *H. dujardini* genome was published alongside a claim of extensive horizontal gene transfer (HGT) from bacteria and other taxa into the tardigrade genome, and a suggestion that HGT might have contributed to the evolution of cryptobiosis [19]. The extensive HGT claim has been robustly challenged [20, 21, 39-41], but the debate as to the contribution of HGT to cryptobiosis remains open. The genomes of these species could be exploited for understanding the mechanisms of rapid-desiccation versus slow-desiccation strategies in tardigrades, the importance of HGT, and the resolution of the deep structure of the Ecdysozoa. However, the available genomes are not of equivalent quality.

We have generated a high quality genome assembly for *H. dujardini*, from an array of data including single-tardigrade sequencing [42] and long, single-molecule reads, and using a heterozygosity-aware assembly method [43, 44]. Gene finding and annotation with extensive RNA-Seq data allowed us to predict a robust gene set. While most (60%) of the genes of *H. dujardini* had direct orthologues in an improved gene prediction for *R. varieornatus*, levels of synteny were very low. We identified an unremarkable proportion of potential horizontal gene transfers. *H. dujardini* showed losses of peroxisome and stress signaling pathways, as described in *R. varieornatus*, as well as additional unique losses. Transcriptomic analysis of anhydrobiosis entry detected higher levels of regulation in *H. dujardini* compared to *R. varieornatus*, as predicted, including regulation of genes with anti-stress and apoptosis functions. Using single copy orthologues, we reanalyzed the position of Tardigrada within Ecdysozoa and found strong support for a Tardigrade+Nematode clade, even when data from transcriptomes of a nematomorph, onychophorans and other ecdysozoan phyla were included. However, rare genomic changes tended to support the traditional Panarthropoda. We discuss our findings in the context of how best to improve genomics of neglected species, the biology of anhydrobiosis and conflicting models of ecdysozoan relationships.

# RESULTS

## THE GENOME OF *H. DUJARDINI*

The genome size of *H. dujardini* has been independently estimated by densitometry to be ~100 Mb [20, 45], but the spans of existing assemblies exceed this, because of contamination with bacterial reads and uncollapsed heterozygosity. We generated new sequencing data (Supplementary Table S1) for *H. dujardini*. Tardigrades, originally purchased in mixed cultures from Sciento, were cultured with a single algal food source. Illumina short reads were generated from a single, cleaned tardigrade [42] and PacBio long single-molecule reads from DNA from a bulk, cleaned tardigrade population (~900,000 animals). We employed an assembly strategy that eliminated evident bacterial contamination [46] and dealt with the 30~60% heterozygosity estimated from k-mer distributions. Our initial Platanus [44] genome assembly had a span of 99.3 Mb in 1,533 contigs, with an N50 length of 250 kb. Further scaffolding and gap filling [47] with PacBio reads and a Falcon [43] assembly of the PacBio reads produced a 104 Mb assembly in only 1,421 scaffolds and an N50 length of 342 kb, N90 count of 343 (Table 1). In comparison with previous assemblies, this assembly has improved contiguity and improved coverage of complete core eukaryotic genes [48, 49]. Read coverage was relatively uniform throughout the genome (Supplementary Figure S1, Supplementary Table S2), with only a few short regions, likely repeats, with high coverage. We identified 29.6 Mb (28.5%) the *H. dujardini* genome as being repetitive (Supplementary Table S3). Simple repeats covered 5.2% of the genome, with a longest repeat unit of 8,943 bp. Seven of the eight longest repeats were of the same repeat unit (GATGGGTTTT)n, were found exclusively at nine scaffold ends and may correspond to telomeric sequence (Supplementary Table S4). The other long repeat was a simple repeat of (CAGA)n and its complementary sequence (GTCT)n, and spanned 3.2 Mb (3% of the genome, longest unit 5,208 bp). We identified eighty-one 5.8S rRNA, two 18S rRNA, and three 28S rRNA loci with RNAmmer [50]. Scaffold0021 contains both 18S and 28S loci, and it is likely that multiple copies of the ribosomal RNA repeat locus have been collapsed in this scaffold, as it has very high read coverage (~5,400 fold, compared to ~113 fold overall, suggesting ~48 copies). tRNAs for each amino acid were found (Supplementary Figure S2) [51]. Analysis of miRNA-Seq data with miRDeep [52] predicted 507 mature miRNA loci (Supplementary Data S1), of which 185 showed similarity with sequences in miRbase [53].

**Table 1 Metrics of *Hypsibius* *dujardini* genome assemblies.** \* The longest scaffolds in the tg assembly are derived from bacterial contaminants.

|  |  |  |  |
| --- | --- | --- | --- |
| Data Source | This work | Edinburgh | UNC |
| Sequencing technologies | Illumina & PacBio | Illumina | Illumina & PacBio |
| Genome version | nHd.3.0 | nHd.2.3 | tg |
| Scaffold number | 1,421 | 13,202 | 16,175 |
| Total Scaffold Length (bp) | 104,155,103 | 134,961,902 | 212,302,995 |
| Average Scaffold Length (bp) | 73,297 | 10,222 | 13,125 |
| Longest Scaffold Length (bp) | 2,115,976 | 594,143 | 1,208,507\* |
| Shortest Scaffold Length (bp) | 1,000 | 500 | 2,002 |
| N50 (bp) (no. scaffs in N50) | 342,180 (#85) | 50,531 (#701) | 17,496 (#3,422) |
| N90 (bp) (no. scaffs in N90) | 65,573 (#343) | 6,194 (#3,280) | 6,637 (#11,175) |
| CEGMA genes found (partial) | 237 (240) | 220 (241) | 221 (235) |
| CEGMA gene duplication ratio | 1.17 (1.23) | 1.35 (1.56) | 3.26 (3.53) |
| Complete BUSCO genes (%) | 93.0 | 92.4 | 88.8 |

We generated RNA-Seq data from active and anhydrobiotic (“tun” stage) tardigrades, and developmental stages of *H. dujardini* (Supplementary Table S1). Gene finding using BRAKER [54] predicted 19,901 genes, with 914 isoforms (version nHd3.0). This set of gene models had higher completeness and lower duplication scores compared to those predicted with MAKER {Holt, 2011 #983}, which uses RNA-Seq and protein evidence (BRAKER :  90.7% MAKER: 77.9% genome based : 86.3%, Metazoan lineage used). Minor manual editing of this gene set to break approximately 40 fused genes generated version nHd3.1. These coding sequence predictions lacked 5’ and 3’ untranslated regions. Mapping of RNA-Seq data to the predicted coding transcriptome showed an average mapping proportion of 50~70%, but the mapping proportion was over 95% against the genome (Supplementary Table S5). A similar mapping pattern for RNA-Seq data to predicted transcriptome was also observed for *R. varieornatus*. Over 70% of the *H. dujardini* transcripts assembled with Trinity [55] mapped to the predicted transcriptome, and a larger proportion to the genome (Supplementary Table S6). RNA-seq reads that are not represented in the predicted coding transcriptome likely derived from UTR regions, unspliced introns or promiscuous transcription. We inferred functional and similarity annotations for ~50% of the predicted proteome (Table 2).

**Table 2 Comparison of the genomes of *H. dujardini* and *R. varieornatus.***\* Uniquely retained ancestral genes include genes shared by only one Tardigrade and at least one non-Tardigrade taxon. \*\* Single-copy Orthologues: orthologues with CDS lengths differing by more than 20% were not considered.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Assembly** | ***H. dujardini* 3.0** | | ***R. varieornatus*1.1** | | **Difference** | |
| **GENOME** | Mb | % | bp | % | Mb | % |
| **Total span** | 104.16 | - | 55.83 | - | 48.33 |  |
| **Genic** | 59.03 | 56.67% | 31.94 | 57.21% | 27.09 | 56.06% |
| exon span | 25.25 | 24.24% | 19.56 | 35.03% | 5.69 | 11.78% |
| intron span | 33.78 | 32.43% | 12.38 | 22.17% | 21.40 | 44.28% |
| **Intergenic** | 45.13 | 43.33% | 23.89 | 42.79% | 21.23 | 43.94% |
| repeat | 27.11 | 26.03% | 10.11 | 18.12% | 17.00 | 35.17% |
| **GENES** | # families | # genes | # families | # genes | #families | #genes |
| number of genes | 11,705 | 19,901 | 9,029 | 13,917 | 2,676 | 5,984 |
| number of proteins (including isoforms) |  | 20,815 |  | 14,538 |  | 6,277 |
| species-specific singletons | 4,364 | 4,364 | 1,995 | 1,995 | 2,369 | 2,369 |
| species-specific gene families | 45 | 258 | 20 | 123 | 25 | 135 |
| shared gene families | 7,296 | 15,279 | 7,014 | 11,799 | 258 | 3,480 |
| uniquely retained ancestral genes \* | 471 | 999 | 189 | 311 | 282 | 688 |
| genes with BLAST matches to SwissProt |  | 8,337 |  | 6,978 |  |  |
| genes with BLAST matches to TrEMBL |  | 10,202 |  | 8,359 |  |  |
| genes with InterPro domain matches |  | 11,227 |  | 8,633 |  |  |
| genes with Gene Ontology terms |  | 7,804 |  | 6,030 |  |  |
| Eukaryote BUSCO completeness (%) |  | 98.1 |  | 97.0 |  |  |
| All genes | mean | median | mean | median | ratio of means | ratio of medians |
| gene length (bp) | 2966 | 2131 | 2295 | 1641 | 1.29 | 1.30 |
| exon span (bp) | 1269 | 978 | 1405 | 1074 | 0.90 | 0.91 |
| exon count (#) | 5.94 | 4 | 6.02 | 4 | 0.99 | 1.00 |
| intron span (bp) | 1697 | 1109 | 889 | 520 | 1.91 | 2.13 |
| intron count (#) | 4.94 | 3 | 5.02 | 3 | 0.98 | 1.00 |
| Single-copy Orthologues \*\* | mean | median | mean | median | ratio of means | ratio of medians |
| gene length (bp) | 3716 | 2776 | 2579 | 1929 | 1.44 | 1.44 |
| exon span (bp) | 1615 | 1278 | 1581 | 1253 | 1.02 | 1.02 |
| exon count (#) | 7.64 | 6 | 6.96 | 6 | 1.10 | 1.00 |
| intron span (bp) | 2101 | 1475 | 998 | 635 | 2.11 | 2.32 |
| intron count (#) | 3716 | 2776 | 2579 | 1929 | 1.44 | 1.44 |

The *H. dujardini* nHd.3.0 genome assembly is available on a dedicated ENSEMBL [56] server, <http://ensembl.tardigrades.org>, where it can be compared with previous assemblies of *H. dujardini* and with the *R. varieornatus* assembly. The ENSEMBL database interface includes an application-programming interface (API) for scripted querying [57]. All data files (including supplementary data files and other analyses) are available from [http://download.tardigrades.org](http://downloads.tardigrades.org), and a dedicated BLAST server is available at <http://blast.tardigrades.org>. All raw data files have been deposited in INSDC databases (NCBI and SRA, Supplementary Table S1) and the assembly (nHd3.1) has been submitted to NCBI under the accession ID MTYJ00000000.

## COMPARISONS WITH *RAMAZZOTTIUS VARIEORNATUS*

We compared this high-quality assembly of *H. dujardini* to that of *R. varieornatus* [22]. In initial comparisons, we noted that *R. varieornatus* had many single-exon loci that had no *H. dujardini* (or other) homologues. Reasoning that this might be a technical artifact, we updated gene models for *R. varieornatus* using BRAKER [54] with additional comprehensive RNA-Seq of developmental stages (Supplementary Table 1). The new prediction included 13,917 protein-coding genes (612 isoforms). This lower gene count compared to the original (19,521 genes) was largely due to a reduction in single-exon genes with no transcript support (from 5,626 in version 1 to 1,777 in the current annotation). Most (12,752, 90%) of the BRAKER-predicted genes were also found in the original set. In both species, some predicted genes may derive from transposons, as 2,474 *H. dujardini* and626 *R. varieornatus* proteins matched Dfam domains [58]. While several of these putatively transposon-derived predictions have a Swiss-Prot [59] homologue (*H. dujardini*: 915, 37%; *R. varieornatus*: 274, 44%), most had very low expression levels.

One striking difference between the two species was in genome size, as represented by assembly span: the *R. varieornatus* assembly had a span of 55 Mb, half that of *H. dujardini* (Table 2). This difference could have arisen through whole genome duplication, segmental duplication, or more piecemeal processes of genome expansion or contraction. *H. dujardini* had 5,984 more predicted genes than *R. varieornatus*. These spanned ~23 Mb, and accounted for about half the additional span. There was no difference in number of exons per gene between orthologues or in the whole predicted gene set. However, comparing orthologues, the intron span per gene in *H. dujardini* was on average twice that in *R. varieornatus* (Figure 1B), and gene length (measured as start codon to stop codon in coding exons) was ~1.3 fold greater in *H. dujardini* (Table 2, Supplementary Figure S3). There was more intergenic noncoding DNA in *H. dujardini*, largely explained by an increase in the repeat content (28.6 Mb in *H. dujardini*, versus 11.1 Mb in *R. varieornatus*).

**Figure 1** **The genomes of *Hypsibius dujardini* and *Ramazzottius varieornatus.* (A) Linkage conservation but limited synteny between *H. dujardini* and *R. varieornatus*.** Whole genome alignment was performed with Murasaki {Popendorf, 2010 #1257}. The left panel shows the whole genome alignment. Similar regions are linked by a line colored following a spectrum based on the start position in *R. varieornatus*. To the right is a re-alignment of the initial segment of *H. dujardini* scaffold0001 (lower), showing matches to several portions of *R. varieornatus* Scaffold0002 (above), illustrating the several inversions that must have taken place. The histograms show pairwise nucleotide sequence identity between these two segments. **(B) Increased intron span in *H. dujardini.*** *H. dujardini* genes are longer because of expanded introns. Frequency histogram of log2 ratio of intron span per gene in 4,728 *H. dujardini* genescompared to their orthologues in *R. varieornatus*. Outliers are defined as genes in *H. dujardini* whose CDS are 20% longer (long outliers; orange; 576 genes) or 20% shorter (short outliers; black; 294 genes) than their orthologues in *R. varieornatus*. **(C)** **Gene neighborhoods conservation between *H. dujardini* and *R. varieornatus*.** To test conservation of gene neighborhoods, we asked whether genes found together in *H. dujardini* were also found close together in *R. varieornatus*. Taking the set of genes on each long *H. dujardini* scaffold, we identified the locations of the reciprocal best BLAST hit orthologues in *R. varieornatus*, and counted the maximal proportion mapping to one *R. varieornatus* scaffold. *H. dujardini* scaffolds were binned and counted by this proportion. As short scaffolds, with fewer genes, might bias this analysis, we performed analyses independently on scaffolds with >10 genes and scaffolds with >20 genes.

Whole genome alignments of *R. varieornatus* and *H. dujardini* using Murasaki [60] revealed a low level of synteny but evidence for conserved linkage at the genome scale, with little conservation of gene order beyond a few loci. For example, comparison of *R. varieornatus* Scaffold002 of with *H. dujardini* scaffold0001 showed linkage, with many orthologous (genome-wide bidirectional best BLAST hit) loci across ~1.7 Mb of the *H. dujardini* genome (Figure 1A). A high proportion of orthologues of genes located on the same scaffold in *H. dujardini* were also in one scaffold in *R. varieornatus*, implying that intrachromosomal rearrangement may be the reason for the low level of synteny (Figure 1C).

We defined protein families in the *H. dujardini* and new *R. varieornatus* predicted proteomes, along with a selection of other ecdysozoan and other animal predicted proteomes (Supplementary Table S7), using OrthoFinder [61], including predicted proteomes from fully-sequenced genomes or predicted proteomes from the fully-sequenced genomes and (likely partial) transcriptomes in two independent analyses. Using these protein families we identified orthologues for phylogenetic analysis, and explored patterns of gene family expansion and contraction, using KinFin [62]. We identified 144,610 protein families in the analysis of 29 fully-sequenced genome species. Of these families, 87.9% were species-specific (with singletons accounting for 11.6% of amino acid span, and multi-protein clusters accounting for 1.2% of span). While only 12.1% of clusters contained members from ≥2 predicted proteomes, they accounted for the majority of the amino acid span (87.2%). *H. dujardini* had more species-specific genes than *R. varieornatus*, and had more duplicate genes in gene families shared with *R. varieornatus* (Table 2). *H. dujardini* also had more genes shared with non-tardigrade outgroups, suggesting loss in *R. varieornatus*. Many families had more members in tardigrades compared to other taxa, and three had fewer members (115 had uncorrected Mann-Whitney U-test probabilities <0.01, but none had differential presence after Bonferroni correction). In nine of the families with tardigrade overrepresentation, tardigrades had more than four times as many members as the average of the other species (Supplementary Data File S2).

There were 1,486 clusters composed solely of proteins predicted from the two tardigrade genomes. Of those, 365 (24.56%) had a congruent domain architecture in both species, including 53 peptidase clusters, 27 kinase clusters and 29 clusters associated with signaling function, including 18 G-protein coupled receptors (see Supplementary Data S3). While these annotations are commonly found in clade-specific families, suggesting that innovation in these classes of function is a general feature in metazoan evolution, of particular interest was innovation in the Wnt signaling pathway. Tardigrade-unique clusters included Wnt, Frizzled and chibby proteins. Of relevance to cryptobiosis, 21 clusters with domain annotation relevant to genome repair and maintenance were synapomorphic for Tardigrada, including molecular chaperones (2), histone/chromatin maintenance proteins (11), genome repair systems (4), nucleases (2) and chromosome cohesion components (2).

## HORIZONTAL GENE TRANSFER IN TARDIGRADE GENOMES

HGT is an interesting but contested phenomenon in animals. Many newly sequenced genomes have been reported to have relatively high levels of HGT, and genomes subject to intense curation efforts tend to have lower HGT estimates. We performed *ab initio* gene finding on the genomes of the model species *Caenorhabditis elegans* and *Drosophila melanogaster* with Augustus [63] and used the HGT index approach [64], which simply classifies loci based on the ratio of their best BLAST scores to ingroup and potential donor taxon databases, to identify candidates. Compared with their mature annotations, we found elevated proportions of putative HGTs in both species (*C. elegans*: 2.09% of all genes, *D. melanogaster*: 4.67%). We observed similarly elevated rates of putative HGT loci, as assessed by the HGT index, in gene sets generated by *ab inito* gene finding in additional arthropod and nematode genomes compared to their mature annotation (Figure 2A, Supplementary Table S8). Thus, the numbers of HGT events found in the genomes of *H. dujardini* and *R. varieornatus* are likely to have been overestimated in these initial, uncurated gene predictions, even after sequence contamination has been removed, as seen in the assembly of Boothby *et al.* [41].

**Figure 2 Horizontal gene transfer in *Hypsibius dujardini.* (A) Horizontal gene transfer ratios in various metazoa*.*** For a set of assembled arthropod and nematode genomes, genes were re-predicted *ab initio* with Augustus. Putative HGT loci were identified using the HGT index for the longest transcript for each gene from the new and the ENSEMBL reference gene sets. In most species, the *ab initio* gene sets had elevated numbers of potential HGT loci compared to their ENSEMBL representations. **(B) Classification of HGT candidates in *H. dujardini*.** Classification of the initial HGT candidates identified in *H. dujardini* by their phylogenetic annotation (prokaryotic, non-metazoan eukaryotic, viral, metazoan and complex), their support in RNA-Seq expression data, and the presence of a homologue in *R. varieornatus*.

Using the HGT index approach [64] we identified 463 genes (2.32% of all genes) as potential HGT candidates in *H. dujardini* (Supplementary Data S4). Using Diamond BLASTX [65], instead of standard BLASTX [66], made only a minor difference in the number of potential HGT events predicted (446 genes, 2.24%). We sifted the initial 463 *H. dujardini* candidates through a series of biological filters (Figure 2B). A true HGT locus will show affinity with its source taxon when analyzed phylogenetically, *i.e.* if a monophyletic clan could be observed in the constructed unrooted tree (a more sensitive test than simple BLAST score ratio), and just under half of the loci (225) were confirmed as HGT events by RAxML [67] analysis of aligned sequences (Figure 2B, Supplementary Data S5). HGT genes are expected to be incorporated into the host genome and to persist through evolutionary time, and 214 of the *H. dujardini* candidates had homologues in *R. varieornatus*, indicating phylogenetic perdurance (Supplementary Data S4 and S5). Of these shared candidates, 113 were affirmed by phylogeny. HGT loci will acquire gene structure and expression characteristics of their host, metazoan genome, and one third (168) of the HGT candidates had RNA-Seq expression values at or above the average for all genes. While metazoan genes usually contain spliceosomal introns, and 367 of the candidate HGT gene models included introns, we regard this a lower-quality validation criterion as gene finding algorithms are programmed to identify introns. Our minimal current estimate for HGT into the genome of *H. dujardini* is 113 genes (0.57% of 19,901 loci) and the upper bound is 463 (2.33%). This is congruent with estimates of 1.58% HGT index derived candidates (of 13,917 genes) for *R. varieornatus* [22].

The putative HGT loci tended to be clustered in the tardigrade genomes, with many gene neighbors of HGT loci also predicted to be HGTs (Supplementary Figure S4). We found 58 clusters of HGT loci in *H. dujardini*, and 14 in *R. varieornatus* (Supplementary Data S6). The largest clusters included up to 6 genes from the same gene family and may have arisen through tandem duplication. These tandem duplication clusters included Intradiol ring-cleavage dioxygenases, UDP glycosyltransferases and alpha/beta fold hydrolases. Several clusters of UDP glycosyltransferases with signatures of HGT from plants were identified in the *H. dujardini* genome, one of which included 6 UDP glycosyltransferases within 12 genes (bHd03905~bHd03916). *H. dujardini* had 40 UDP glycosyltransferase genes, 29 of which were classified as glucuronosyltransferase (UGT, K00699) by KEGG ORTHOLOG mapping with KAAS {Moriya, 2007 #22}, and of these 27 were HGT candidates. While UGT can function in a number of pathways, we found that the whole ascorbate synthesis pathway, in which UGT metabolizes UDP-D-glucuronate to D-Glucuronate, has been acquired by HGT in *H. dujardini*. *R. varieornatus* has only acquired L-gulonolactone oxidase (Supplementary Figure S5). Gluconolactonase and L-gluonolactone oxidase were consistently expressed at low levels (10~30 TPM), but L-ascorbate degradation enzyme L-ascorbate oxidase was not expressed (TPM<1).

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## THE GENOMICS OF ANHYDROBIOSIS IN TARDIGRADES

We explored the *H. dujardini* proteome and the reannotated *R. varieornatus* proteome for loci implicated in anhydrobiosis. In the new *R. varieornatus* proteome, we found 16 CAHS loci and 13 SAHS loci and one copy each of MAHS, RvLEAM and Dsup. In *H. dujardini* we identified 12 CAHS loci, 10 SAHS loci and single members of the RvLEAM and MAHS families (Supplementary Table S9). Direct interrogation of the *H. dujardini* genome with *R. varieornatus* loci identified an additional possible CAHS-like locus and an additional SAHS-like locus. We found no evidence for a *H. dujardini* homologue of Dsup. Phylogenetic analyses revealed a unique duplication of CAHS3 in *R. varieornatus*. No SAHS2 orthologue was found in *H. dujardini* (Supplementary Figure S6), and most of the *H. dujardini* SAHS loci belonged to a species-specific expansion that was orthologous to a single *R. varieornatus* SAHS locus, RvSAHS13. SAHS1-like genes in *H. dujardini* and SAHS1- and SAHS2-like genes in *R. varieornatus* were locally duplicated, forming SAHS clusters on single scaffolds.

*R. varieornatus* was reported to have undergone extensive gene loss in the stress responsive transducer of mTOR pathway and in the peroxisome pathway, which generates H2O2 during the beta-oxidation of fatty lipids. *H. dujardini* was similarly compromised (Figure 3A). We identified additional gene losses in the peroxisome pathway in *H. dujardini*, as peroxisome proteins PEK5, PEK10, and PEK12, while present in *R. varieornatus*, were not found in *H. dujardini* (TBLASTN search against genome with E-value threshold of 1E-3).

**Figure 3 The genomics of anhydrobiosis in tardigrades. (A) Gene losses in Hypsibiidae.** Gene losses were detected by mapping to KEGG pathways using KAAS, and validated by BLAST TBLASTN search of KEGG orthologue gene amino acid sequences. Light blue and gray boxes indicate genes conserved and lost in both tardigrades, respectively. Furthermore, purple boxes represent genes retained in only one species, and red as genes that have been detected as HGT. Numbers on the top right of boxes indicates copy numbers of multiple copy genes in *H. dujardini.* Genes annotated as CASP3 and CDC25A have contradicting annotation with KAAS and Swiss-Prot, however the KAAS annotation was used. **(B)** **Differential gene expression in tardigrades on entry to the anhydrobiotic state.** The TPM expression for each sample was calculated using Kallisto, and the fold change between active and tun, and the TPM expression in the tun state were plotted. Genes that likely contribute to anhydrobiosis were colored,. Genes that had an orthologue in the other species are plotted as circles; other genes are plotted as triangles.

To identify gene functions associated with anhydrobiosis, we explored differential gene expression in both species in fully hydrated and post-desiccation samples from both species. We compared single individual RNA-Seq of *H. dujardini* undergoing anhydrobiosis [42] with new data for *R. varieornatus* induced to enter anhydrobiosis in two ways: slow desiccation (~24 h) and fast desiccation (~30 min). Successful anhydrobiosis was assumed when >90% of the samples prepared in the same chamber recovered after rehydration. Many more genes were differentially upregulated by entry into anhydrobiosis in *H. dujardini* (1,422 genes, 7.1%) than in *R. varieornatus* (fast desiccation: 64 genes, 0.5%; slow desiccation: 307 genes, 2.2%) (Supplementary Data File S6). The fold change distribution of the whole transcriptome of *H. dujardini* (mean 8.33, median 0.91±69.90 SD) was significantly broader than those of both fast (0.67, 0.48±2.25) and slow (0.77, 0.65±0.79) desiccation *R. varieornatus* (U-test, p-value <0.001) (Figure 3B).

For the loci differentially expressed in anhydrobiosis (Supplementary Data S7), we investigated their membership of gene families with elevated numbers in tardigrades and functional annotations associated with anhydrobiosis. Proteins with functions related to protection from oxidants, such as SOD and peroxiredoxin, were found to have been extensively duplicated in tardigrades. In addition, the mitochondrial chaperone (BSC1), osmotic stress related transcription factor NFAT5, and apoptosis related gene PARP families were expanded in tardigrades. Chaperones were extensively expanded in *H. dujardini* (HSP70, DnaK, and DnaJ subfamily C-5, C-13, B-12), and the DnaJ subfamily B3, B-8 was expanded in *R. varieornatus*. In *H. dujardini,* we found five copies of DNA repair endonuclease XPF, which functions in the nucleotide-excision repair pathway, and, in *R. varieornatus*, four copies of the double-stranded break repair protein MRE11 (as reported previously [22]) and additional copies of DNA ligase 4, from the non-homologous end-joining pathway. In both *R. varieornatus* [22] and *H. dujardini* some of the genes with anhydrobiosis-related function appear to have been acquired through HGT. All copies of catalase were high-confidence HGTs, and one copy was differentially expressed during *H. dujardini* anhydrobiosis (expression rises from 0 TPM to 27.5 TPM during slow dehydration in *H. dujardini*). *R. varieornatus* had eleven trehalase loci (nine trehalases and two acid trehalase-like proteins). While *H. dujardini* did not have an orthologue of trehalose-6-phosphatase synthase (TPS), a gene required for trehalose synthesis, *R. varieornatus* had a HGT-derived TPS (Supplementary Figure S5). Previous studies in *Milnesium tardigradum* have shown that trehalose does not accumulate during anhydrobiosis, and this is supported by the low expression of the *R. varieornatus* TPS gene (10-20 TPM in active and tun states). We note that the *R. varieornatus* TPS had highest similarity to TPS from bacterial species in Bacteriodetes, including *Chitinophaga*, which was one of the contaminating organisms in the Boothby *et al.* dataset. The *R. varieornatus* locus contains spliceosomal introns that do not shatter the TPS protein sequence, and is surrounded by metazoan-affinity loci. The ascorbate synthesis pathway appears to have been acquired through HGT in *H. dujardini*, and a horizontally acquired L-gulonolactone oxidase was identified in *R. varieornatus* (Supplementary Figure S5).

Several protection-related genes were differentially expressed in anhydrobiotic *H. dujardini*, including CAHS (8 loci of 15), SAHS (2 of 10), RvLEAM (1 of 1), and MAHS (1 of 1). Loci involved in reactive oxygen protection (five SOD genes, six glutathione-S transferase genes, a catalase gene, and a LEA gene) were upregulated under desiccation. Interestingly, two trehalase loci were upregulated, even though we were unable to identify any TPS loci in *H. dujardini*. We also identified differentially expressed transcription factors that may regulate anhydrobiotic responses. Two calcium-signaling factors, calmodulin (CaM) and a cyclic nucleotide gated channel (CNG-3), were both upregulated, which may drive cAMP synthesis through adenylate cyclase. Although *R. varieornatus* is capable of rapid anhydrobiosis induction, complete desiccation is unlikely to be as rapid in natural environments, and regulation of gene expression under slow desiccation might reflect a more likely scenario. Fitting this expectation, five CAHS loci and a single SAHS locus were upregulated after slow desiccation, but none were differentially expressed following rapid desiccation. Most *R. varieornatus* CAHS and SAHS orthologues had high expression in the active state, several over 1,000 TPM. In contrast, *H. dujardini* CAHS and SAHS orthologues had low resting expression (median 0.7 TPM), and were upregulated (median 1916.8 TPM) on anhydrobiosis induction. Aquaporins contribute to transportation of water molecules into cells, and could be involved in anhydrobiosis [68]. Aquaporin-10 was highly expressed in *R. varieornatus* and differentially expressed in anhydrobiotic *H. dujardini*. *M. tardigradum* has at least ten aquaporin loci [69], *H. dujardini* has eleven, and *R. varieornatus* ten. The contributions to anhydrobiosis of additional genes identified as upregulated (including cytochrome P450, several solute carrier families, and apolipoproteins) are unknown.

Some genes differentially expressed in both *H. dujardini* and *R. varieornatus* slow-desiccation anhydrobiosis were homologous (Supplementary Data S9). Of the 1,422 differentially expressed genes from *H. dujardini*, 121 genes were members of 70 protein families that also contained 115 *R. varieornatus* differentially expressed genes. These included CAHS, SAHS, glutathione-S transferase, and SOD gene families, but in each case *H. dujardini* had more differentially expressed copies than *R. varieornatus*. Other differentially expressed gene families were annotated as metalloproteinases, calcium binding receptors and G-protein coupled receptors, suggesting that these functions may participate in cellular signaling during induction of anhydrobiosis. Many more (887) gene families included members that were upregulated by anhydrobiosis in *H. dujardini* but unaffected by desiccation in *R. varieornatus*. These gene families included 1,879 *R. varieornatus* genes, some (154) were highly expressed in the active state (TPM >100).

In addition to gene loss, we predicted that the tardigrades might have undergone expansion in gene families active in anhydrobiotic physiology. We identified three gene families, each containing members with significant differential expression during anhydrobiosis, which had elevated numbers of members in the tardigrades compared to the other taxa analysed. *H. dujardini* and *R. varieornatus* had more members of OG000684 (33 and 8, respectively) than any other (mode of 1 and mean of 1.46 copies in the other 28 species, with a maximum of 4 in the moth *Plutella xylostella*). Proteins in OG000684 were annotated with domains associated with ciliar function. OG0002660 contained three proteins from each of *H. dujardini* and *R. varieornatus*, but a mean of 1.2 from other species. OG0002660 was annotated as fumarylacetoacetase, which acts in phenylalanine metabolism. Fumarylacetoacetase has been identified as a target of the SKN-1 induced stress responses in *C. elegans* [70]. OG0002103 was also overrepresented in the tardigrades (3 in each species), while 23 of the other species had 1 copy. Interestingly the extremophile nematode *Plectus murrayi* had 4 copies. OG0002103 was annotated as GTP cyclohydrolase, involved in formic acid metabolism, including tetrahydrobioterin synthesis. Tetrahydrobioterin is a cofactor of aromatic amino acid hydroxylases, which metabolize phenylalanine. The association of these functions with anhydrobiosis merits investigation.

## PHYLOGENETIC RELATIONSHIPS OF TARDIGRADA

From the two analyses of protein families shared between *H. dujardini*, *R. varieornatus*, taxa from other ecdysozoan phyla, and two lophotrochozoan outgroup taxa (one that included only taxa with whole genome data, and a second that also included taxa with transcriptome data) we selected putative orthologous protein families. These were screened to eliminate evident paralogous sequences, and alignments were concatenated into a supermatrix. The genomes-only supermatrix included 322 loci from 28 taxa spanning 67,256 aligned residues, and had 12.5% missing data. The alignment was trimmed to remove low-quality regions. The genomes and transcriptomes supermatrix included 71 loci from 37 taxa spanning 68,211 aligned residues, had 27% missing data, and was not trimmed. Phylogenomic analyses were carried out in RAxML (using the General Time Reversible model with Gamma distribution of rates model, GTR+G) and PhyloBayes (using a GTR plus rate categories model, GTR-CAT+G). We also explored bipartition support from individual gene trees, and RAxML and Phylobayes analyses of 6-state Dayhoff recoded amino acid alignments using the GTR model (as GTR-CAT cannot be used on these recoded data; Supplementary Data S8).

The genomes-only phylogeny (Figure 4A) strongly supported Tardigrada as a sister to monophyletic Nematoda. Within Nematoda and Arthropoda, the relationships of species were congruent with previous analyses, and the earliest branching taxon in Ecdysozoa was Priapulida. RAxML bootstrap and PhyloBayes Bayes proportion support was high across the phylogeny, with only two internal nodes in Nematoda and Arthropoda receiving less-than-maximal support. Analysis of individual RAxML phylogenies derived from the 322 loci revealed a degradation of support deeper in the tree, with 53% of trees supporting a monophyletic Arthropoda, 56% supporting Tardigrada plus Nematoda, and 54% supporting the monophyly of Arthropoda plus Tardigrada plus Nematoda. The phylogeny derived from the genomes and transcriptomes dataset (Figure 4B) also recovered credibly resolved Nematoda and Arthropoda, and, as expected, placed Nematomorpha as sister to Nematoda. Tardigrada was again recovered as sister to Nematoda plus Nematomorpha, with maximal support. Priapulida plus Kinorhyncha was found to arise basally in Ecdysozoa. Unexpectedly, Onychophora, represented by three transcriptome datasets, was sister to an Arthropoda plus (Tardigrada, Nematomorpha, Nematoda) clade, again with high support.

**Figure 4** **Phylogeny of Ecdysozoa. (A)** Phylogeny of 28 species from 5 phyla, based on 322 loci derived from whole genome sequences, and rooted with the lophotrochozoan outgroup. Labels on nodes are Bayes proportions from PhyloBayes analysis / bootstrap proportions from RAxML maximum likelihood bootstraps / proportion of trees of individual loci supporting each bipartition. Note that different numbers of trees were assessed at each node, depending on representation of the taxa at each locus. \* indicates maximal support (Bayes proportion of 1.0 or RAxML bootstrap of 1.0). **(B)** Phylogeny of 36 species from 8 phyla, based on 71 loci derived using PhyloBayes from whole genome and transcriptome sequences, and rooted with the lophotrochozoan outgroup. All nodes had maximal support in Bayes proportions and RAxML bootstrap, except those labeled (Bayes proportion, \*= 1.0 / RAxML bootstrap).

## RARE GENOMIC CHANGES AND TARDIGRADE RELATIONSHIPS

We tested support for a Nematoda+Tardigrada clade in rare genomic changes [71] in core developmental gene sets and protein family evolution. Rare genomic changes can be used as strong parsimony markers of phylogenetic relationships that are hard to resolve using model-based sequence analyses. An event shared by two taxa can be considered to support their relationship where the likelihood of the event is *a priori* expected to be vanishingly small.

HOX genes are involved in anterior-posterior patterning across the Metazoa, with a characteristic set of paralogous genes present in most animal genomes, organized as a tightly regulated cluster. The ancestral cluster is hypothesized to have included HOX1, HOX2, HOX3, HOX4, HOX5, and a HOX6-8 like locus and HOX9. The HOX6-8 and HOX9 types have undergone frequent, independent expansion and contraction during evolution, and HOX clustering has broken down in some species. HOX complements are generally conserved between related taxa, and gain and loss of HOX loci can be considered a rare genomic change. We surveyed HOX loci in tardigrades and relatives (Figure 5A). In the priapulid *Priapulus caudatus* nine HOX loci have been described [72], but no HOX6-8/*AbdA*-like gene was identified. All arthropods surveyed (including representatives of the four classes) had a complement of HOX loci very similar to that of *D. melanogaster*, with at least ten loci including HOX6-8 and HOX9. Some HOX loci in some species have undergone duplication, particularly HOX3/*zen*. In the mite *Tetranychus urticae* and the salmon louse *Lepeoptheirius salmonis* we identified “missing” HOX genes in the genome. For Onychophora, the sister group to Arthropoda, HOX loci have only been identified through PCR screens [73, 74], but they appear to have the same complement as Arthropoda.

**Figure 5 The position of tardigrada in ecdysozoa. (A)** **HOX genes in tardigrades and other Ecdysozoa.** HOX gene losses in Tardigrada and Nematoda. HOX gene catalogues of tardigrades and other Ecdysozoa were collated by screening ENSEMBL Genomes and WormBase Parasite. HOX orthology groups are indicated by different colours. Some “missing” HOX loci were identified by BLAST search of target genomes (indicated by vertical striping of the affected HOX). “?” indicates that presence/absence could not be confirmed because the species was surveyed by PCR or transcriptomics; loci identified by PCR or transcriptomics are indicated by a dotted outline. “X” indicates that orthologous HOX loci were not present in the genome of that species. Some species have duplications of loci mapping to one HOX group, and these are indicated by boxes with dashed outlines. The relationships of the species are indicated by the cladogram to the left, and circles on this cladogram indicate Dollo parsimony mapping of events of HOX group loss on this cladogram. Circles are coloured congruently with the HOX loci. **Evolution of gene families under different hypotheses of tardigrade relationships. (B)** Tardigrades share more gene families with Arthropoda than with Nematoda. In this network, derived from the OrthoFinder clustering at inflation value 1.5, nodes represent species (0: *Anopheles gambiae*, 1: *Apis mellifera*, 2: *Acyrthosiphon pisum*, 3: *Ascaris suum*, 4: *Brugia malayi*, 5: *Bursaphelenchus xylophilus*, 6: *Caenorhabditis elegans*, 7: *Cimex lectularius*, 8: *Capitella teleta*, 9: *Dendroctonus ponderosae*, 10: *Daphnia pulex*, 11: *Hypsibius dujardini*, 12: *Ixodes scapularis*, 13: *Meloidogyne hapla*, 14: *Nasonia vitripennis*, 15: *Octopus bimaculoides*, 16: *Priapulus caudatus*, 17: *Pediculus humanus*, 18: *Plectus murrayi*, 19: *Pristionchus pacificus*, 20: *Plutella xylostella*, 37: *Ramazzottius varieornatus*, 22: *Solenopsis invicta*, 23: *Strigamia maritima*, 24: *Tribolium castaneum*, 25: *Trichuris muris*, 26: *Trichinella spiralis*, 27: *Tetranychus urticae*, 38: *Drosophila melanogaster*). The thickness of the edge connecting two nodes is weighted by the count of shared occurrences of both nodes in OrthoFinder-clusters. Links involving *H. dujardini* (red) and *R. varieornatus* (orange) are coloured. The inset box on the lower right shows the average weight of edges between each phylum and both Tardigrades, normalized by the maximum weight (*i.e.* count of co-occurrences of Tardigrades and the annelid *C. teleta*)" **(C)** Gene family birth synapomorphies at key nodes in Ecdysozoa under two hypotheses: Tardigrada+Nematoda *versus* Tardigrada+Arthropoda. Each graph shows the number of gene families at the specified node inferred using Dollo parsimony from OrthoFinder clustering at inflation value 1.5. Gene families are grouped by the proportion of taxa above that node that contain a member. Note that to be included as a synapomorphy of a node, a gene family must contain proteins of at least one species of each child node of the node in question, and thus there are no synapomorphies with <0.3 proportional proteome coverage in Nematoda and <0.2 in Arthropoda, and all synapomorphies of Tardigrada have 1.0 representation. **(D)** Gene family birth synapomorphies for Tardigrada+Arthropoda (grey) and Tardigrada+Nematoda (yellow) for OrthoFinder clusterings performed at different MCL inflation parameters.

In *H. dujardini*, a reduced HOX gene complement (six genes in five orthology groups) has been reported [75], and we confirmed this reduction using our improved genome (Figure 5A). The same, reduced complement was also found in the genome of *R. varieornatus* [22], and the greater contiguity of the *R. varieornatus* genome shows that five of the six HOX loci are on one large scaffold, distributed over 2.7 Mb, with 885 non-HOX genes separating them. The *H. dujardini* loci were unlinked in our assembly, except for the two HOX9/*AbdB*-like loci, and lack of gene level synteny precludes ordering of these scaffolds based on the *R. varieornatus* genome. The order of the HOX genes on the *R. varieornatus* scaffolds is not colinear with other, unfragmented clusters, as HOX6-8/*ftz* and the pair of HOX9/*AbdB* genes are inverted, and HOX4/*dfd* is present on a second scaffold (and not found between HOX3 and HOX6-8/*ftz* as would be expected).

The absences of HOX2/*pb,* HOX5/*scr* and HOX6-8/*Ubx/AbdA* in both tardigrade species is reminiscent of the situation in Nematoda, where these loci are also absent [76-78]. HOX gene evolution in Nematoda has been dynamic. No Nematode HOX2 or HOX5 orthology group genes were identified, and only a few species had a single HOX6-8 orthologue. Duplication of the HOX9/*AbdB* locus was common, generating, for instance the *egl-5, php-3* and *nob-1* loci in *Caenorhabditis* species. The maximum number of HOX loci in a nematode was seven, deriving from six orthology groups. Loss of HOX3 happened twice (in *Syphacia muris* and in the common ancestor of Tylenchomorpha and Rhabditomorpha). The independent loss in *S. muris* was confirmed in two related pinworms*, Enterobius vermicularis* and *Syphacia oblevata*. The pattern of presence and absence of the *Antp*-like HOX6-8 locus is more complex, requiring six losses (in the basally-arising enoplean *Enoplis brevis*, the chromadorean *Plectus sambesii*, the pinworm *Syphacia muris*, the ancestor of Tylenchomorpha, the diplogasteromorph *Pristionchus pacificus*, and the ancestor of *Caenorhabditis*). We affirmed loss in the pinworms by screening the genomes of *E. vermicularis* and *S. oblevata* as above, and no HOX6-8/*Antp*-like locus is present in any of the over 20 genomes available for *Caenorhabditis*. A PCR survey for HOX loci and screening of a *de novo* assembled transcriptome from the nematomorph *Paragordius varius* identified six putative loci from five HOX groups. The presence of a putative HOX2/*pb*-like gene suggests that loss of HOX2 may be independent in Tardigrada and Nematoda.

Gene family birth can be used as another rare genomic marker. We analyzed the whole proteomes of ecdysozoan taxa for gene family births that supported either the Tardigrada+Nematoda model or the Tardigrada+Arthropoda (*i.e.* Panarthropoda) model. We mapped gene family presence and absence across the two contrasting phylogenies using KinFin [62] using different inflation parameters in the MCL step in OrthoFinder (Supplementary Data S10). Using the default inflation value of 1.5 the two tardigrades shared more gene families with Arthropoda than they did with Nematoda (Figure 5B). The number of gene family births synapomorphic for Arthropoda and Nematoda were identical under both phylogenies, as was expected (Table 3; Figure 5C; Supplementary Data S11). Many synapomorphic families had variable presence in the daughter taxa of the common ancestors of Arthropoda and Nematoda, likely because of stochastic gene loss or lack of prediction. However, especially in Nematoda, most synapomorphic families were present in a majority of species (Figure 5C).

**Table 3 Gene family births that support different relationships of Tardigrada**. \* Protein families from OrthoFinder clustering at inflation value 1.5. \*\* Domain annotations are reported where proteins from more than one third of the proteomes in the family had that annotation. IPR = InterPro domain identifier; PF = PFam identifier.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Protein Family\*** | **Number of proteins** | **Proportion of proteomes represented** | | | | **Domain annotations\*\*** |
| **all** | **Nematoda (n=9)** | **Arthropoda (n=15)** | **Tardigrada (n=2)** |
| **Synapomorphies with membership ≥ 0.7 under the Panarthropoda (Tardigrada + Arthropoda) hypothesis** | | | | | | |
| **OG0000436** | 104 | 1.00 | *0.00* | 1.00 | 1.00 | Serine proteases, trypsin domain (IPR001254) |
| **OG0001236** | 54 | 1.00 | *0.00* | 1.00 | 1.00 | Major facilitator superfamily associated domain (IPR024989) |
| **OG0002592** | 36 | 1.00 | *0.00* | 1.00 | 1.00 | Spätzle (IPR032104) |
| **OG0006538** | 19 | 1.00 | *0.00* | 1.00 | 1.00 | Leucine-rich repeat (IPR001611) |
| **OG0006541** | 19 | 1.00 | *0.00* | 1.00 | 1.00 | None |
| **OG0006869** | 17 | 1.00 | *0.00* | 1.00 | 1.00 | Thioredoxin domain (IPR013766) |
| **OG0005117** | 27 | 0.88 | *0.00* | 0.93 | 0.50 | BTB/POZ domain (IPR000210) |
| **OG0005941** | 22 | 0.77 | *0.00* | 0.73 | 1.00 | None |
| **OG0006662** | 18 | 0.82 | *0.00* | 0.80 | 1.00 | None |
| **OG0006889** | 17 | 0.71 | *0.00* | 0.73 | 0.50 | None |
| **OG0006940** | 17 | 0.82 | *0.00* | 0.87 | 0.50 | EGF-like domain (IPR000742), Laminin G domain (IPR001791) |
| **OG0006941** | 17 | 0.71 | *0.00* | 0.67 | 1.00 | EF-hand domain (IPR002048) |
| **OG0006951** | 17 | 0.71 | *0.00* | 0.67 | 1.00 | Adipokinetic hormone (IPR010475) |
| **OG0007141** | 16 | 0.82 | *0.00* | 0.80 | 1.00 | None |
| **OG0007285** | 15 | 0.71 | *0.00* | 0.67 | 1.00 | GPCR, family 2, secretin-like (IPR000832) |
| **OG0007290** | 15 | 0.82 | *0.00* | 0.80 | 1.00 | Allatostatin (IPR010276) |
| **OG0007298** | 15 | 0.88 | *0.00* | 0.87 | 1.00 | None |
| **OG0007328** | 15 | 0.71 | *0.00* | 0.67 | 1.00 | Sulfakinin (IPR013259) |
| **OG0007463** | 14 | 0.77 | *0.00* | 0.73 | 1.00 | Peptidase S1A, nudel (IPR015420), Serine proteases, trypsin domain (IPR001254), Low-density lipoprotein (LDL) receptor class A repeat (IPR002172) |
| **OG0007689** | 13 | 0.71 | *0.00* | 0.67 | 1.00 | Marvel domain (IPR008253) |
| **Synapomorphies with membership ≥ 0.7 under the Tardigrada + Nematoda hypothesis** | | | | | | |
| **OG0005423** | 26 | 0.82 | 0.89 | *0.00* | 0.50 | Amidinotransferase (PF02274) |
| **OG0006414** | 20 | 0.82 | 0.78 | *0.00* | 1.00 | Proteolipid membrane potential modulator (IPR000612) |
| **OG0007199** | 16 | 0.91 | 1.00 | *0.00* | 0.50 | Zona pellucida domain (IPR001507) |
| **OG0007812** | 13 | 0.82 | 0.78 | *0.00* | 1.00 | None |
| **OG0008368** | 11 | 0.82 | 0.78 | *0.00* | 1.00 | RUN domain (IPR004012) |

At inflation value 1.5, we found six gene families present that had members in both tardigrades and all 14 arthropods under Panarthropoda, but no gene families were found in both tardigrades and all 9 nematodes under the Tardigrada+Nematoda hypothesis (Supplemental Table 10). Allowing for stochastic absence, we inferred 154 families to be synapomorphic for Tardigrada+Arthropoda under the Panarthropoda hypothesis, and 99 for Tardigrada+Nematoda under the Tardigrada+Nematoda hypothesis (Figure 5D). More of the Tardigrada+Arthropoda synapomorphies had high species representation than did the Tardigrada+Nematoda synapomorphies. This pattern was also observed in analyses using different inflation values and in analyses including the transcriptome from the nematomorph *Paragordius varius*.

We explored the biological implications of these putative synapomorphies by examining the functional annotations of each protein family that contained members from ≥70% of the ingroup species (Table 3). Under Tardigrada+Arthropoda twenty families had ≥70% of the ingroup taxa represented, and six were universally present. These included important components of developmental and immune pathways, neuromodulators and others. Two families were annotated as serine endopeptidases, one missing in some arthropods that included *D. melanogaster* Nudel, and one found in all species. Another synapomorphic family, found in all species, included *spätzle* orthologues. Spätzle is a cysteine-knot, cytokine-like ligand involved in dorso-ventral patterning, and is the target of a serine protease activation cascade initiated by Nudel protease. The identification of more than one member of a single regulatory cascade as potential gene family births suggests that the pathway may have been established in a Tardigrada+Arthropoda most recent common ancestor. Other Tardigrada+Arthropoda-synapomorphic families were annotated with ommatidial apical extracellular matrix (eyes shut), adipokinetic hormone, neuromodulatory allatostatin-A, drosulfakinin, leucine-rich repeat, thioredoxin, major facilitator superfamily associated, and domain of unknown function DUF4728 domains. However, nine of the twenty Panarthropoda synapomorphic families had no informative domain annotations. Under Tardigrada+Nematoda, only five putatively synapomorphic families had members from ≥70% of the ingroup species. Four of these had domain matches (proteolipid membrane potential modulator, zona pellucida, RUN and amidinotransferase domains), and one contained no proteins with identifiable domains.

# DISCUSSION

## A ROBUST ESTIMATE OF THE *HYPSIBIUS DUJARDINI* GENOME

We have sequenced and assembled a high-quality genome for the tardigrade *H. dujardini*, utilizing new data, including single-molecule, long-read sequencing, and heterozygosity-aware assembly methods. Comparison of genomic metrics with previous assemblies for this species showed that our assembly is more complete and more contiguous than has been achieved previously, and retains minimal uncollapsed heterozygous regions. The span of this new assembly is much closer to independent estimates of the size of the *H. dujardini* genome (75 - 100 Mb) using densitometry and staining. The *H. dujardini* genome is thus nearly twice the size of that of the related tardigrade *R. varieornatus*. We compared the two genomes to identify differences that would account for the larger genome in *H. dujardini*. While *H. dujardini* had ~6,000 more protein coding genes than *R. varieornatus*, these accounted for only ~23 Mb of the additional span, and are not obviously simple duplicates of genes in *R. varieornatus*. Analyses of the gene contents of the two species showed that while *H. dujardini* had more species-specific genes, it also had greater numbers of loci in species-specific gene family expansions than *R. varieornatus*, and had lost fewer genes whose origins could be traced to a deeper ancestor. *H. dujardini* genes had, on average, the same structure (~6 exons per gene) as did *R. varieornatus,* however introns in *H. dujardini* genes were on average twice the length of their orthologues in *R. varieornatus* (255 bases versus 158 bases). Finally, the *H. dujardini* genome was more repeat rich (28.5% compared to only 21% in *R. varieornatus*). These data argue against simple whole genome duplication in *H. dujardini*. The genome of *H. dujardini* is larger because of expansion of non-coding DNA, including repeats and introns, and acquisition and retention of more new genes and gene duplications than *R. varieornatus*. The disparity in retention of genes with orthologues outside the Tardigrada, where *R. varieornatus* has lost more genes than has *H. dujardini*, suggests that *R. varieornatus* may have undergone genome size reduction, and that the ancestral tardigrade (or hypsibiid) genome is more likely to have been ~100 Mb than 54 Mb. We await additional tardigrade genomes with interest. While we identified linkage between genes in the two tardigrades, local synteny was relatively rare. In this these genomes resemble those of the genus *Caenorhabditis*, where extensive, rapid, within-chromosome rearrangement has served to break close synteny relationships while, in general, maintaining linkage [79]. The absence of chromosomal level assemblies for either tardigrade (and lack of any genetic map information) precludes definitive testing of this hypothesis.

## HORIZONTAL GENE TRANSFER IN TARDIGRADES: *H. DUJARDINI* HAS A NORMAL METAZOAN GENOME

Boothby *et al.* made the surprising assertion that 17.5% of *H. dujardini* genes originated through HGT from a wide range of bacterial, fungal and protozoan donors [19]. Subsequently, several groups including our teams proved that this finding was the result of contamination of their tardigrade samples with cobionts, and less-than-rigorous screening of HGT candidates [20, 21, 39, 40]. We found that the use of uncurated gene-finding approaches yielded elevated HGT proportion estimates in many other nematode and arthropod genomes, even where contamination is unlikely to have been an issue. It is thus essential to follow up initial candidate sets of HGT loci with detailed validation. We screened our new *H. dujardini* assembly for evidence of HGT, identifying 3.7% of the protein coding genes as initial potential candidates. After careful assessment using phylogenetic analysis and expression evidence, we identified a maximum of 2.3% and a high-confidence set of only 0.6% of *H. dujardini* genes that could have originated through HGT. HGT was also much reduced (~1.6%) in the high-quality *R. varieornatus* genome. These proportions are congruent with similar analyses of *C. elegans* and *D. melanogaster*[64]. Curation of the genome assemblies and gene models may decrease the proportion further.

Tardigrades do not have elevated levels of HGT in their genomes, but some HGT events are of importance in anhydrobiosis. All *H. dujardini* catalase loci were of bacterial origin, as described for *R. varieornatus* [22]. While trehalose phosphatase synthase was absent from *H. dujardini*, *R. varieornatus* has a TPS that likely was acquired by HGT (Supplementary Data S5). As *M. tardigradum* does not have a TPS homologue, while other ecdysozoan taxa do, this suggests that TPS may have been lost in the common ancestor of eutardigrada and regained in *R. varieornatus* by HGT after divergence from *H. dujardini*.

## CONTRASTING MODES OF ANHYDROBIOSIS IN TARDIGRADES

Genes with likely roles in protection against extreme stress previously identified in *R. varieornatus* were largely conserved in *H. dujardini*. Both CAHS and SAHS families had high copy numbers in both species, with independent expansions. However, we did not find a Dsup orthologue in *H. dujardini*. *H. dujardini* has similar gene losses to *R. varieornatus* in pathways that produce ROS and in cellular stress signaling pathways, which suggest that the gene losses occurred before the divergence of the two species. This loss of important signaling pathway genes may disconnect signals of stress induction from activating downstream response systems, such as cell cycle regulation, transcription and replication inhibition, and apoptosis, that must be suppressed if anhydrobiosis is to be achieved successfully. As cellular protection and repair pathways were highly conserved, damaged cell systems will still be protected and repaired. Indeed some stress-related gene families had undergone gene family expansion in one or both tardigrades. SOD was duplicated in both species, as was a calcium activated potassium channel, which has been implicated in cellular signaling during anhydrobiosis [23]. The elevated gene family expansion in *H. dujardini* compared to *R. varieornatus* may be related to retention and expansion of induced stress response systems.

The transcriptome response to anhydrobiosis differs between the two tardigrades. *H. dujardini* has an induced transcriptomic response where *R. varieornatus* does not. We found that *H. dujardini* had more genes differentially expressed on anhydrobiosis than *R. varieornatus*. As anticipated, more *R. varieornatus* loci were differentially expressed when desiccated at a slow pace. Genes induced by slow desiccation included CAHS and SAHS genes, and anti-oxidant related genes. Although most of these genes were highly expressed (>100 TPM) in the active state, the induction of these genes may enable higher recovery. CAHS and SAHS loci were also over-expressed on anhydrobiosis in *H. dujardini*. We found a variety of calcium related transporters and receptors were differentially expressed on anhydrobiosis. Kondo *et al.* suggested that cellular signaling using calmodulin and calcium may be required for anhydrobiosis [23], but it is still unclear how this is related to anhydrobiosis. Calcium, and other metal ion, concentrations could be increased during dehydration, and thus could act as a desiccation signal. Trehalose is known for its role in protecting cellular systems from dehydration [35, 36, 80, 81]. It has been hypothesized that it may not be required for tardigrade anhydrobiosis, as trehalose was not detected in *Milnesium tardigradum* [31]. Trehalose synthesis via TPS has been lost in *H. dujardini,* although we found a HGT-origin TPS in R. varieornatus. Unexpectedly, three *R. varieornatus* trehalase loci were differentially expressed on slow desiccation, including two with over 200 TPM in the anhydrobiotic state. As trehalose degradation should not be required in the absence of trehalose, there may be an alternative pathway for trehalose synthesis.

## THE POSITION OF TARDIGRADES IN THE METAZOA

Our phylogenomic analyses found Tardigrada, represented by *H. dujardini* and *R. varieornatus* genomes as well as transcriptomic data from *Milnesium tardigradum* and *Echiniscus testudo*, to be sisters to Nematoda, not Arthropoda. This finding was robust to inclusion of additional phyla, such as Onychophora and Nematomorpha*,* and to filtering the alignment data to exclude poorly represented or rapidly evolving loci. This finding is both surprising, and not new. Many previous molecular analyses have found Tardigrada to group with Nematoda, whether using single genes or ever larger gene sets derived from transcriptome and genome studies [1-3]. This phenomenon has been attributed to long branch attraction in suboptimal datasets, with elevated substitutional rates or biased compositions in Nematoda and Tardigrada mutually and robustly driving Bayesian and Maximum Likelihood algorithms to support the wrong tree. Strikingly, in our analyses including taxa for which transcriptome data are available, we found Onychophora to lie outside a ((Nematoda, Nematomorpha, Tardigrada), Arthropoda) clade. This finding, while present in some other analyses (*e.g.* component phylogenies summarized in [2]), conflicts with accepted systematic and many molecular analyses. We note that Onychophora was only represented by transcriptome datasets, and that there is accordingly an elevated proportion of missing data in the alignment for this phylum.

That a tree linking Tardigrada with Nematoda is “wrong” is a prior supported by developmental and anatomical data. Tardigrades are segmented, have appendages, and have a central and peripheral nervous system anatomy that can be homologized with those of Onychophora and Arthropoda [82, 83]. In contrast, nematodes are unsegmented, have no lateral appendages and have a simple nervous system. The myoepithelial triradiate pharynx, found in Nematoda, Nematomorpha, and Tardigrada, is one possible morphological link, but Nielsen has argued persuasively that the structures of this organ in nematodes and tardigrades (and other taxa) are distinct and thus non-homologous [5].

*H. dujardini* has a reduced complement of HOX loci, as does *R. varieornatus*. Some of the HOX loci missing in the Tardigrada are the same as those absent from Nematoda. Whether these absences are a synapomorphy for a Nematode-Tardigrade clade, or simply a product of homoplasious evolution remains unclear. It may be that miniaturisation of Nematoda and Tardigrada during adaptation to life in interstitial habitats facilitated the loss of specific HOX loci involved in post-cephalic patterning, and that both nematodes and tardigrades can be thought to have evolved by reductive evolution from a more fully featured ancestor. It may be intrinsically easier to lose some HOX loci than others. While tardigrades retain obvious segmentation, nematodes do not, with the possible exception of repetitive cell lineages along the anterior-posterior axis during development [84]. We note that until additional species were analyzed, the pattern observed in *C. elegans* was assumed to be the ground pattern for all Nematoda. More distantly related Tardigrada may have different HOX gene complements to these hypsibiids, and a pattern of staged loss similar to that in Nematoda [76-78] may be found.

Assessment of gene family births as rare genomic changes lent support to a Tardigrada+Arthropoda clade, but the support was not striking. There were more synapomorphic gene family births when a Tardigrada+Arthropoda (Panarthropoda) clade was assumed than when a Tardigrada+Nematoda clade was assumed. However, analyses under the assumption of Tardigrada+Nematoda identified synapomorphic gene family births at 50% of the level found when Panarthropoda was assumed. We note that recognition of gene families may be compromised by the same “long branch attraction” issues that plague phylogenetic analyses, and also that any taxon where gene loss is common (such as has been proposed for Nematoda as a result of its simplified body plan) may score poorly in gene family membership metrics. The short branch lengths that separate basal nodes in the analysis of the panarthropodan-nematode part of the phylogeny of Ecdysozoa may make robust resolution very difficult. We explored the biological implications of the synapomorphies that supported Panarthropoda by examining the functional annotations of each protein family (Supplementary Table S10) and were surprised that many of these deeply conserved loci have escaped experimental, genetic or biochemical annotation. One family included *spätzle*, a cysteine-knot, cytokine-like family involved in dorso-ventral patterning as well as immune response, and two others were serine endopeptidases, including *nudel*, which is part of the same pathway as *spätzle*. This pathway may be a Panarthropod innovation. Thus our analyses of rare genomic changes lent some support to the Panarthropoda hypothesis, as did analysis of miRNA gene birth [2], but analysis of HOX loci may conflict with this.

The question of tardigrade relationships remains open [4]. While we found support for a clade of Tardigrada, Onychophora, Arthropoda, Nematoda and Nematomorpha, the branching order within this group remains contentious, and in particular the positions of Tardigrada and Onychophora are poorly supported and/or variable in our and others’ analyses. Full genome sequences of representatives of Onychophora, Heterotardigrada (the sister group to the Eutardigrada including Hypsibiidae), Nematomorpha and enoplian, basally arising Nematoda are required. Resolution of the conflicts between morphological and molecular data will be informative, either of the ground state of a nematode-tardigrade ancestor, or of the processes that drive homoplasy in rare genomic changes and robust discovery of non-biological trees in sequence-based phylogenomic studies.

# METHODS

## TARDIGRADE CULTURE AND SAMPLING

The tardigrade *Hypsibius dujardini* Z151 was purchased from Sciento (Manchester, UK). *H. dujardini* Z151 and *Ramazzottius varieornatus* strain YOKOZUNA-1 were cultured as previously described [24, 42]. Briefly, tardigrades were fed *Chlorella vulgaris* (Chlorella Industry) on 2% Bacto Agar (Difco) plates prepared with Volvic water, incubated at 18 °C for *H. dujardini* and 22 °C for *R. varieornatus* under constant dark conditions. Culture plates were renewed every 7~8 days. Anhydrobiotic adult samples were isolated on 30 µM filters (Millipore), and placed in a chamber maintained at 85% relative humidity (RH) for 48 hr for *H. dujardini*, and 30% RH for 24 hr and additional 24 hr at 0% RH for slow-dried *R. varieornatus*, and 0% RH for 30 min on a 4 cm x 4 cm Kim-towel with 300 µL of distilled water, and additional 2 hr without the towel for fast-dried *R. varieornatus*. Successful anhydrobiosis was assumed when >90% of the samples prepared in the same chamber recovered after rehydration.

## SEQUENCING

Genomic DNA for long read sequencing was extracted using MagAttract HMW DNA Kit (Qiagen) from approximately 900,000 *H. dujardini*. DNA was purified twice with AMPure XP beads (Beckman Coulter). A 20 kb PacBio library was prepared following the manual “20 kb Template Preparation Using BluePippin Size-Selection System (15 kb Size Cutoff)” (PacBio SampleNet - Shared Protocol) using SMARTBell Template Prep Kit 1.0 (Pacific Biosciences), and was sequenced using 8 SMRT Cells Pac V3 with DNA Sequencing Reagent 4.0 on a PacBio RSII System (Pacific Biosciences) at Takara Bio Inc. Briefly, purified DNA was sheared, targeting 20 kb fragments, using a g-TUBE (Covaris). Following end-repair and ligation of SMRTbell adapters, the library was size-selected using BluePippin (Sage Science) with a size cut-off of 10 kb. The size distribution of the library was assayed on TapeStation 2200 (Agilent) and quantified using the Quant-iT dsDNA BR Assay Kit (Invitrogen). MiSeq reads from a single *H. dujardini* individual (DRR055040) and HiSeq reads are from our previous reports [20, 21].

For mRNA-Seq to be used in genome annotation, 30 individuals were collected from each of the following conditions in three replicates: active and dried adults (slow dried for *R. varieornatus*), eggs (1, 2, 3, 4, 5, 6 and 7 days after laying) and juveniles (1, 2, 3, 4, 5, 6 and 7 days after hatching). Due to sample preparations, *R. varieornatus* juveniles were sampled until juvenile 1st day. For gene expression analysis, we sampled 2~3 individuals of fast-dried *R. varieornatus*. All mRNA-Seq analyses were conducted with 3 replicates. Specimens were thoroughly washed with Milli-Q water on a sterile nylon mesh (Millipore), immediately lysed in TRIzol reagent (Life Technologies) using three cycles of immersion in liquid nitrogen followed by 37°C incubation. Total RNA was extracted using the Direct-zol RNA kit (Zymo Research) following the manufacturer’s instructions, and RNA quality was checked using the High Sensitivity RNA ScreenTape on a TapeStation (Agilent Technologies). For library preparation, mRNA was amplified using the SMARTer Ultra Low Input RNA Kit for Sequencing v.4 (Clontech), and Illumina libraries were prepared using the KAPA HyperPlus Kit (KAPA Biosystems). Purified libraries were quantified using a Qubit Fluorometer (Life Technologies), and the size distribution was checked using the TapeStation D1000 ScreenTape (Agilent Technologies). Libraries size selected above 200 bp by manually excision from agarose were purified with a NucleoSpin Gel and PCR Clean-up kit (Clontech). The samples were then sequenced on the Illumina NextSeq 500 in High Output Mode with a 75-cycle kit (Illumina) as single end reads, with 48 multiplexed samples per run. Adapter sequences were removed, and sequences were demultiplexed using the bcl2fastq v.2 software (Illumina). For active and dried adults, RNA-Seq was also conducted starting from approximately 10,000 individuals, similarly washed but RNA extraction with TRIzol reagent (Life Technologies) followed by RNeasy Plus Mini Kit (Qiagen) purification. Library preparation and sequencing was conducted at Beijing Genomics Institute.

For miRNA-Seq, 5,000 individuals were homogenized using Biomasher II (Funakoshi), and TRIzol (Invitrogen) was used for RNA extraction, and purified by isopropanol precipitation. Size selection  of fragments of 18-30 nt using electrophoresis, preparation of the sequencing library for Illumina HiSeq 2000 and subsequent  (single end) sequencing was carried out by  Beijing Genomics Institute .

All sequence data were validated for quality using FastQC [85].

## GENOME ASSEMBLY

The MiSeq reads from whole genome amplified DNA were merged with Usearch [86] and both merged and unmerged pairs were assembled with SPAdes [87] as single-end. The SPAdes assembly was checked for contamination with BLAST+ BLASTN [88] against the nr [89] database and no observable contamination was found with blobtools [46]. Illumina data from Boothby *et al.* [19] were mapped to the SPAdes assembly with Bowtie2 [90] and read pairs were retained if at least one of them mapped to the assembly. These reads were then assembled, scaffolded and gap closed with Platanus [44]. The Platanus assembly was further scaffolded and gap closed using the PacBio data with PBJelly [91].

Falcon [43] assembly of PacBio data was performed on the DNAnexus platform. Using this Falcon assembly, Platanus assembly was extended using SSPACE-LongReads [47], and gap-filled with PBJelly [91] with default parameters. Single-individual MiSeq reads were mapped to the assembly with BWA, and all contigs with coverage < 1, length <1000 bp, or those corresponding to the mitochondrial genome were removed. At this stage, one CEGMA gene became unrecognized by CEGMA [48] probably due to multiple PBJelly runs, and therefore the contig harboring that missing CEGMA gene was corrected by Pilon [92] using the single individual MiSeq reads. We also validated genomic completeness with BUSCO using the eukaryote linage.

## GENE FINDING

mRNA-Seq data (Development, Active-tun 10k animals) were mapped to the genome assembly with TopHat [90, 93] without any options. Using the mapped data from TopHat, BRAKER [54] was used with default settings to construct a GeneMark-ES [94] model and an Augustus [63] gene model, which are used for *ab initio* prediction of genes. The getAnnotFasta.pl script from Augustus was used to extract coding sequences from the GFF3 file. Similarly, to construct a modified version of the *R. varieornatus* genomes annotation, we used the development and anhydrobiosis (Supplementary Table S1) RNA-Seq data for BRAKER annotation. We found that a few genes were mis-annotated (MAHS in both species, a CAHS orthologue in *R. varieornatus*), due to fusion with a neighboring gene, and these were manually curated. tRNA and rRNA genes were predicted with tRNAscan-SE [51] and RNAmmer [50], respectively. BUSCO was used again to validate the completeness of the predicted gene set for both tardigrades.

The mRNA-Seq data used to predict the gene models were mapped with BWA MEM [95] against the predicted CDS sequences, the genome, and a Trinity [55] assembled transcriptome. We also mapped the mRNA-Seq data used for gene expression analysis (single individual *H. dujardini* and fast/slow dry of *R. varieornatus*) of the active state and tun state. After SAM to BAM conversion and sorting with SAMtools view and sort [96], we used QualiMap [97] and bedtools [98] for mapping quality check.

To annotate the predicted gene models, we performed similarity searches using BLAST BLASTP [66] against Swiss-Prot, TrEMBL [59], and HMMER hmmsearch [99] against Pfam-A [100] and Dfam [58], KAAS analysis for KEGG orthologue mapping [101], and InterProScan [102] for domain annotation. We used RepeatScout [103] and RepeatMasker [104] for *de novo* repeat identification. To compare *H. dujardini* gene models to those of *R. varieornatus*, we also ran BLAST BLASTP searches against the updated *R. varieornatus* proteome, and TBLASTN search against the *R. varieornatus* genome and extracted bidirectional best hits with in-house Perl scripts.

For miRNA prediction we used miRDeep [52] to predict mature miRNA within the genome, using the mature miRNA sequences in miRBase [53]. The predicted mature miRNA sequences were then searched against miRBase with ssearch36 [105] for annotation by retaining hits with identity > 70% and a complete match of bases 1-7, 2-8 or 3-9.

## 

## HORIZONTAL GENE TRANSFER ANALYSES

HGT genes were identified using the HGT index approach [64]. Swiss-Prot and TrEMBL were downloaded [59], and sequences with “Complete Proteome” in the Keyword were extracted. Following the method of Boschetti *et al.*, an Arthropoda-less and Nematoda-less database was constructed. These databases were searched with DIAMOND [65] using as query all CDS sequences, using the longest transcript for each gene (DIAMOND BLASTX). Hits with an E-value below 1e-5 were kept. The HGT index (Hu) was calculated as Bo - Bm, the bit score difference between the best non-metazoan hit (Bo) and the best metazoan hit (Bm), and genes with Hu ≥ 30 were identified as HGT candidates.

To assess if *ab initio* annotation of genomes biases the calculation of the HGT index, we calculated HGT indices for genomes in ENSEMBL-Metazoa [56] that had corresponding Augustus [63] gene models and ran *ab initio* gene prediction. We analysed *Aedes aegypti*, *Apis mellifera*, *Bombus impatiens*, *Caenorhabditis brenneri*, *C. briggsae*, *C. elegans,* *C. japonica*, *C. remanei*, C*ulex quinquefasciatus*, *Drosophila ananassae,* *D. erecta*, *D. grimshawi*, *D. melanogaster*, *D. mojavensis*, *D. persimilis*, *D. pseudoobscura*, *D. sechellia*, *D. simulans*, *D. virilis*, *D. willistoni*, *D. yakuba*, *Heliconius melpomene*, *Nasonia vitripennis*, *Rhodnius prolixus*, *Tribolium castaneum*, and *Trichinella spiralis*. Gene predictions for each organism were conducted using autoAugPred.pl from the Augustus package with the corresponding model (Supplementary Table S8). The longest isoform sequence for all genes were extracted for both ENSEMBL and *ab initio* annotations, and the HGT index was calculated for each gene in all organisms. To assess if using DIAMOND BLASTX biases HGT index calculation, we ran BLAST BLASTX [66] searches with *H. dujardini*, and calculated the HGT index using the same pipeline.

The blast-score based HGT index provided a first-pass estimate of whether a gene had been horizontally transferred from a non-metazoan species. Phylogenetic trees were constructed for each of the 463 candidates selected on the HGT index along with their best BLAST hits as described above (Supplementary Data S4). Protein sequences for the BLAST hits were aligned along with the HGT candidate using MAFFT [106]. RAxML [67] was used to build individual trees (from 461 candidates, as 2 of the protein sets had less than 4 sequences and trees could not be built for them). HGT candidates were categorized as deriving from prokaryotes, viruses, metazoans, and non-metazoan eukaryotes based on the monophyletic clades that they were placed in. Any that could not be classified monophyletically were classified as complex. OrthoFinder [61] with default BLAST+ BLASTP search settings and an inflation parameter of 1.5 was used to identify orthogroups containing *H. dujardini* and *R. varieornatus* protein-coding genes. These orthogroups were used to identify the *R. varieornatus* HGT homologues of *H. dujardini* HGT candidates. HGT candidates were classified as having high gene expression levels if they had an average gene expression greater than the overall average gene expression level of 1 TPM.

## ANHYDROBIOSIS ANALYSES

To identify genes responsive to anhydrobiosis, we explored transcriptome (Illumina mRNA-Seq) data for both *H. dujardini* and *R. varieornatus*. Individual mRNA-Seq data for *H. dujardini* [42] before and during anhydrobiosis were contrasted with new sequence data for *R. varieornatus* similarly treated. We mapped the mRNA-Seq reads to the coding sequences of the relevant species with BWA MEM [95] and after summarizing the read count of each gene, we used DESeq2 [107] for differential expression calculation, using false discovery rate (FDR) correction. Genes with a FDR below 0.05, an average expression level (in transcripts per kilobase of model per million mapped fragments; TPM) of over 1, and a fold change over 2 were defined as differentially expressed genes. Gene expression (TPM) was calculated with Kallisto [108], and was parsed with custom Perl scripts. To assess if there were any differences in fold change distributions, we used R to calculate the fold change for each gene ( anhydrobiotic / (active+0.1) ), and conducted a U test using the wilcox.text() function. We mapped the differentially expressed genes to KEGG pathway maps [109] to identify pathways that were likely to be differentially active during anhydrobiosis.

## PROTEIN FAMILY ANALYSES and COMPARATIVE GENOMICS

For comparison with *R. varieornatus*, we first aligned the genomes of *H. dujardini* and *R. varieornatus* with Murasaki, and visualized with gmv [60]. The lower tf-idf anchor filter was set to 500. A syntenic block was observed between scaffold0001 of *H. dujardini* and scaffold002 of *R. varieornatus*. We extracted the corresponding regions (*H. dujardini*: scaffold0001 363,334-2,100,664, *R. varieornatus*: scaffold002 2,186,607-3,858,816), and conducted alignment with Mauve [110]. We determined the number of bidirectional best hit (BBH) orthologues on the same scaffold in both *H. dujardini* and *R. varieornatus*. We extracted gene pairs that had an identity of more than 90% by ClustalW2 [102], and calculated the identity of first and last exon between pairs. Tardigrade-specific, protection-related genes (CAHS, SAHS, MAHS, RvLEAM, Dsup) were identified by BLASTP, and were subjected to phylogenetic analysis using Clustalw2 [102] and FastTree [111], and visualized with FigTree [112].

HOX loci were identified using BLAST, and their positions on scaffolds and contigs assessed. To identify HOX loci in other genomes, genome assembly files were downloaded from ENSEMBL Genomes [56] or Wormbase ParaSite [113, 114] and formatted for local search with BLAST+ [88]. Homeodomain alignments were generated using Clustal Omega [115] and phylogenies estimated with RAxML [67] to classify individual homeodomains.

Protein predictions from genomes of Annelida (1 species), Nematoda (9), Arthropoda (15), Mollusca (1), Priapulida (1) were retrieved from public databases (Supplementary Table S7). Proteomes were screened for isoforms (Supplementary Data S12) and longest isoforms were clustered with the proteins of *H. dujardini* and *R. varieornatus* using OrthoFinder 1.1.2 [61] at different inflation values (Supplementary data S10). Proteins from all proteomes were functionally annotated using InterProScan [102]. OrthoFinder output was analyzed using KinFin [62] under two competing phylogenetic hypotheses: either “Panarthropoda”, where Tardigrada and Arthropoda share a more recent common ancestor distinct from Nematoda or where Tardigrada and Nematoda share a more recent common ancestor distinct from Arthropoda (see Supplementary Data S11 for input files used in KinFin analysis). Enrichment and depletion in clusters containing proteins from Tardigrada and other taxa was tested using a two-sided Mann-Whitney-U test of the count (if at least two taxa had non-zero counts) and results were deemed significant at a p-value threshold of p=0.01.

A graph-representation of the OrthoFinder clustering (at Inflation value = 1.5) was generated using the generate\_network.py script distributed with KinFin. The nodes in the graph were positioned using the ForceAtlas2 layout algorithm implemented in Gephi.

Single-copy orthologues between *H. dujardini* and *R. varieornatus* were identified using the orthologous groups defined by OrthoFinder. Using the Ensembl Perl API, gene structure information (gene lengths, exon counts and intron spans per gene) were extracted for these gene pairs. To avoid erroneous gene predictions biasing observed trends, *H. dujardini* genes which were 20% longer or 20% shorter were considered outliers.

## PHYLOGENOMICS

The whole-genome OrthoFinder clustering at inflation value 1.5 was mined for potential single-copy orthologues for phylogenetic analysis. Transcriptome data were retrieved for additional tardigrades (2 species), a priapulid (1), kinorhynchs (2) and onychophorans (3) (Supplementary Table S11). Assembled transcripts for *Echiniscus testudo, Milnesium tardigradum, Pycnophyes kielensis* and *Halicryptus spinulosus* were downloaded from the NCBI Transcriptome Shotgun Assembly (TSA) Database. EST sequences of *Euperipatoides kanangrensis, Peripatopsis sedgwicki* and *Echinoderes horni* were download from NCBI Trace Archive and assembled using CAP3 [116]. Raw mRNA-seq reads for *Peripatopsis capensis* were downloaded from NCBI SRA, trimmed using skewer [117] and assembled with Trinity [55]. Protein sequences were predicted from all transcriptome data using TransDecoder [118], retaining a single open reading frame per transcript. Predicted proteins from these transcriptomes were used along with the genome-derived proteomes in a second OrthoFinder clustering analysis.

We identified putatively orthologous genes in the OrthoFinder clusters for the genome and the genome-plus-transcriptome datasets. For both datasets the same pipeline was followed. Clusters with 1-to-1 orthology were retained. For clusters with median per-species membership equal to 1 and mean less than 2.5, a phylogenetic tree was inferred with RAxML (using the LG+G model). Each tree was visually inspected to identify the largest possible monophyletic clan, and in-paralogues and spuriously included sequences were removed. Individual alignments of each locus were filtered using trimal [119]  and then concatenated into a supermatrix using fastconcat [120]. The supermatrices were analysed with RAxML [67] with 100 ML bootstraps and PhyloBayes [121] (see Supplementary Table S11 for specific commands). Trees were summarized in FigTree.

## DATABASING AND DATA AVAILABILITY

All raw data have been deposited in the relevant INSDC databases. The *H. dujardini* assembly (nHd3.1) has been deposited at DDBJ/ENA/GenBank under the accession MTYJ00000000. All mRNA-Seq data have been uploaded to GEO and SRA under the accession IDs GSE94295 and SRP098585, and the PacBio raw reads and miRNA-Seq data into SRA under the accession IDs SRX2495681 and SRX2495676. Accession IDs for each individual sequence file are given in Supplementary Table S1. We have established a dedicated Ensembl genome browser (version 85) [56] using the EasyImport pipeline [122] and imported the *H. dujardini* genome and annotations described in this paper and the new gene predictions for *R. varieornatus*. These data are available to browse, query and download at <http://www.tardigrades.org>.

## SOFTWARE USAGE AND DATA MANIPULATION

We used open source software tools where available, as detailed in Supplementary Table S11. Custom scripts developed for the project are uploaded to https://github.com/abs-yy/Hypsibius\_dujardini\_manuscript. We used G-language Genome Analysis Environment [123, 124] for sequence manipulation.

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# Author Contributions

YY, GDK, DRL, LS, SK, MB performed informatics analyses, DDH found the conditions for effective anhydrobiosis, KI sequenced small RNAs, MT managed the computational resources, GK, KA performed sequencing and assembly, TK provided the *Ramazzottius varieornatus* genome. All members participated in writing the manuscript. MB and KA supervised the project.

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