The transcriptome of the swimbladder-nematode Anguillicola crassus: Resources for an alien parasite

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Abstract

Background: *Anguillicola crassus* is an ecologically, economically and evolutionary interesting nematode. It has been introduced from Asia, where it parasitises the Japanese eel *Angilla japonica*, to Europe 30 years ago. Today it infects stocks of the endangered, commercially exploited European eel *Anguilla anguilla*, permitting and necessitating research in a newly established host-parasite system. Furthermore phylogenetics places *A. crassus* at a key position for the emergence of parasitism, basal to one of the major clades of parasitic nematodes.

Results: After extensive screening of 756.363 raw pyrosequencing reads, we assembled 353.055 into 11.371 contigs spanning 7.971.550 bases and additionally obtained 21.147 singleton and lower quality contigs spanning 8.095.986 bases. We obtained annotations for ca. 60% of the contigs and 40% of the tentatively unique genes (TUGs) confirming the high quality of especially the contigs. We identified 5112 high quality single nucleotide polymorphisms (SNPs) and suggest 199 of them as most suitable markers for population-genetic studies. The correlation between different analyses provided further insights and confirmed biologicaly relevant expectations: we found an overabundance of predicted signal peptide cleavage sites in sequence conserved in Nematoda and novel in *A. crassus*, correlations between coding polymorphism and differential expression, between coding polymorphism and peptide cleavage sites and between conservation and presence of ortholgs with lethal

rnai-phenotypes in *C. elegans*. GO-term analysis identified an enrichment of peptidases and subunits of the respiratory chain for transcritps under positive selection. Enzymes for energy metabolism were also found enriched in genes differentially expressed between European and Asian *A. crassus*.

Conclusions: The transcriptome of *A. crassus* is a basis for molecular research on this important species. It furthermore provides unique insights into the evolution of parasitism in the Spirurina. Energy metabolism differs between European and Asian worms due to modification in the different host environment or divergent evolution of gene expression.

Background

The nematode Anguillicola crassus Kuwahara, Niimi et Itagaki, 1974 [1] is a parasite of freshwater eels of the genus Anguilla, and adults localise to the swim bladder where they feed on blood. Larvae are transmitted via crustacean intermediate hosts [2]. Originally endemic to East-Asian populations of the Japanese eel (Anguilla japonica), *A. crassus* has attracted interest due to recent anthropogenic expansion of its geographic and host ranges to Europe and the European eel (Anguilla anguilla). Recorded for the first time in 1982 in North-West Germany [3], where it was most likely introduced through the live-eel trade [4, 5], *A. crassus* has spread rapidly through populations of its newly acquired host [6]. At the present day it is found in all *An. anguilla* populations except those in Iceland [7]. *A. crassus* can be regarded as a model for invasive parasite introduction and spread [8].

A. crassus has a major impact on *An. anguilla* populations. In its natural host in Asia infection prevalence and mean intensity of infection are lower than in Europe [9], where high prevalence (above 70% [10]) and high infection intesities have been reported throughout the newly colonized area [11]. The virulence of A .crassus in this new host has been attributed to an inadequate immune response in *An. anguilla* [12]. While the An. japonica is capable of killing larvae of the parasite after vaccination [13] or under high infection pressure [14], responses in *An. anguilla* have hallmarks of pathology, including thickening of the swim bladder wall [15]. Interestingly host also affects the adult size and life-history of the nematodes: In European eels the nematodes are bigger and develop and reproduce faster [16].

The genus Anguillicola is placed in the nematode suborder Spirurina (clade III sensu [17]) [18, 19]. The

Spirurina are exclusively parasitic and include important human pathogens (the causative agents of filariases and ascariasis) as well as prominent veterinary parasites. Molecular phylogenetic analyses place Anguillicola in a clade of spirurine nematodes (Spirurina B of [Laetsch et al submitted]) that have an freshwater or marine intermediate host, but infect a wide range of carnivorous definitive hosts. Spirurina B is sister to the main Spirurina C, including the agents of filariases and ascariasis), and thus *A. crassus* may be used as an outgroup taxon to understand the evolution of parasitic phenotypes in these species. Recent advances in sequencing technology (often termed Next Generation Sequencing; NGS), provide the opportunity for rapid and cost-effective generation of genome-scale data. The Roche 454 platform [20] offers longer reads than other NGS technologies, and thus is suited to de novo assembly of genome-scale data in previously understudied species. Roche 454 data has particular application in transcriptomics [21]. The difference in the biology of *A. crassus* in *An. japonica* (coevolved) and *An. anguilla* (recently captured) eel hosts likely results from an interaction between different host and parasite responses, underpinned by definitive differences in host genetics, and possible genetic differentiation between the invading European and endemic Asian parasites. As part of a programme to understand the invasiveness of *A. crassus* in *An. anguilla*, we are investigating differences in gene expression and genetic distinction between invading European and endemic Asian *A. crassus* exposed to the two different host species. Here we report on the generation of a reference transcriptome for *A. crassus* based on Roche 454 data, and explore patterns of gene expression and diversity.

Methods

Nematode samples, RNA extraction, cDNA synthesis and Sequencing

A. crassus from *An. japonica* were sampled from Kao-Ping river and an adjacent aquaculture in Taiwan as described in [14]. Worms from *An. anguilla* were sampled in Sniardwy Lake, Poland (53.751959N, 21.730957E) and from the Linkenheimer Altrhein, Germany (49.0262N, 8.310556E). After determination of the sex of adult nematodes, they were stored in RNA-later (Quiagen, Hilden, Germany) until extraction of RNA. RNA was extracted from individual adult male and female nematodes and from a population of L2 larvae (Table 1). RNA was reverse transcribed and amplified into cDNA using the MINT-cDNA synthesis kit (Evrogen, Moscow, Russia). For host contamination screening a liver-sample from an uninfected *An. japonica* was also processed. Emulsion PCR was performed for each cDNA library according to the manufacturer's protocols (Roche/454 Life Sciences), and sequenced on a Roche 454 Genome Sequencer FLX. All samples were sequenced using the FLX Titanium chemistry, except for the taiwanese female

sample T2, which was sequenced using FLX standard chemistry, to generate between 99,000 and 209,000 raw reads. For the L2 larval library, which had a larger number of non-*A. crassus*, non-*Anguilla* reads, we confirmed that these data were not laboratory contaminants by screening Roche 454 data produced on the same run in independent sequencing lanes.

Trimming, quality control and assembly

Raw sequences were extracted in `fasta`-format (with the corresponding qualities files) using `sffinfo` (Roche/454) and screened for adapter sequences of the MINT-amplification-kit using `cross-match` [22] (with parameters `-minscore 20 -minmatch 10`). `Seqclean` [23] was used to identify and remove poly-A-tails, low quality, repetitive and short (<100 base) sequences. All reads were compared to a set of screening databases using `BLAST` (expect value cutoff E<1e-5, low complexity filtering turned off: -F F). The databases used were (a) a host sequence database comprising an assembly of the *An. japonica* Roche 454 data, a unpublished assembly of *An. anguilla* Sanger dideoxy sequenced expressed sequence tags (made available to us by Gordon Cramb, University of St Andrews) and transcripts from EeelBase [24] a publicly available transcriptome database for the European eel; (b) a database of ribosomal RNA (rRNA) sequences from eel species derived from our Roche 454 data and EMBL-Bank; and (c) a database of rRNA sequences identified in our *A. crassus* data by comparing the reads to known nematode rRNAs from EMBL-Bank. This last database notably also contained xenobiont rRNA sequences. Reads with matches to one of these databases over more than 80% of their length and with greater than 95% identity were removed from the dataset. Screening and trimming information was written back into sff-format using `sfffile` (Roche 454). The filtered and trimmed data were assembled using the combined assembly approach [21]: Two assemblies were generated, one using `Newbler v2.6` [20] (with parameters `-cdna -urt`), the other using `Mira v3.2.1` [25] (with parameters `-job=denovo,est,accurate,454`). The resulting two assemblies were combined into one using `Cap3` [26] at default settings and contigs were labeled by whether they derived from both assemblies or one assembly only (for a detailed analysis of the assembly categories see the supporting Methods file).

Post-assembly classification and taxonomic assignment of contigs

After assembly contigs were assessed a second time for host and other contamination by comparing them (using `BLAST`) to the three databases defined above, and also to nembase4, a nematode transcriptome database derived from whole genome sequencing and EST assemblies [27, 28]. For each contig, the

highest-scoring match was recorded as long as it spanned more than 50% of the contig. We also compared the contigs to the NCBI non-redundant nucleotide (NCBI-nt) and protein (NCBI-nr) databases, recording the taxonomy of all best matches with expect values better than 1e-05. TUGs with a best hit to non-Metazoans and to Chordata within Metazoa were additionally excluded from further analysis.

Protein prediction and annotation

Protein translations were predicted from the contigs using `prot4EST` (version 3.0b) [29]. Proteins were predicted either by joining single high scoring segment pairs (HSPs) from a `BLAST` search of uniref100 [30], or by `ESTscan` [31], using as training data the *Brugia malayi* complete proteome back-translated using a codon usage table derived from the `BLAST` HSPs, or, if the first two methods failed, simply the longest ORF in the contig. For contigs where the protein prediction required insertion or deletion of bases in the original sequence, we also imputed an edited sequence for each affected contig. Annotations with Gene Ontology (GO), Enzyme Commission (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms were inferred for these proteins using `Annot8r` (version 1.1.1) [32], using the annotated sequences available in uniref100 [30]. Up to 10 annotations based on a `BLAST` similarity bitscore cut-off of 55 were obtained for each annotation set. The complete *B. malayi* proteome (as present in uniref100) and the complete *C. elegans* proteome (as present in wormbase v.220) were also annotated in the same way. `SignalP V4.0` [33] was used to predict signal peptide cleavage sites and signal anchor signatures for the *A. crassus*-transcriptome and similarly again for the proteomes of the tow model-worms. Additionally `InterProScan` [34] (command line utility `iprscan` (version 4.6) with options `-cli -format raw -iprlookup -seqtype p -goterms`) was used to obtain domain based annotations for the high credibility assembly (highCA) derived contigs.

We recorded the presence of a lethal rnai-phenotype in the *C. elegans* ortholog of each TUG using the biomart-interface [35] to wormbase v. 220 through the R-package `biomaRt` [36].

Single nucleotide polymorphism analysis

We mapped the raw reads against the the complete set of contigs, replacing imputed sequences for originals where relevant, using `ssaha2` [37] (with parameters `-kmer 13 -skip 3 -seeds 6 -score 100 -cmatch 10 -ckmer 6 -output sam -best 1`). From the `ssaha2` output, pileup-files were produced using `samtools` [38], discarding reads mapping to multiple regions. `VarScan` [39] (`pileup2snp`) was used with default parameters on pileup-files to output lists of single nucleotide polymorphisms (SNPs) and their

locations. For enrichment analysis of GO-terms we used the R-package `GOstats` [40].
Using `Samtools` [38] (`mpileup -u`) and `Vcftools` [41] (`view -gcv`) we genotyped individual libraries for the list of previously found overall SNPs. Genotype-calls were accepted at a phred-scaled genotype quality threshold of 10. In addition to the relative heterozygosity (number of homozygous sites/number of heterozygous sites) we used the R package `Rhh` [42] to calculate internal relatedness [43], homozygosity by loci [44] and standardized heterozygosity [45] from these data.
Using 1000 bootstrap replicates we confirmed the significance of heterozygote-heterozygote correlation by analyzing the mean and 95% confidence intervals from 1000 bootstrap replicates estimated for all measurements.

Gene-expression analysis

Read-counts were obtained from the `bam`-files generated also for genotyping using the R-package `Rsamtoools` [46]. Counts to off target data and lowCA contigs were disregarded. Furthermore contigs with less than 32 reads over all libraries were excluded from analysis, to avoid inference based on too low overall experssion values. Because very low coverage from library E1 and L2 leading highly variable normalized data, we excluded these libraries from analysis.
The statistic of Audic and Claverie [47] as implemented in `ideg6` [48] was used to contrast single libries. Differential expression between libraries from different sex of worms was accepted for genes differeing between all female libraries E2, T1 and T2 versus the male (M) library (p <0.01) but not within any of the female libraries at the same threshold. Differential expression between libraries from European and Asian origin was accepted for genes differeing between libraries E2 versus T1 and T2 (p <0.01) but not between T1 versus T2.

Over-representation analyses

Prior to analysis of GO-term over-representation (based on dn/ds or expression values) we used the R-package `annotationDbi` [49] to obtain a full list of associations (also with higer-level terms) from `annot8r`-annotations. We then used the R-package `topGO` [50] to traverse the annotation-graph and analyse each node in the annotation for over-representation of the associated term in the focal gene-set compared to a appropriate universal gene-set (all contigs with dn/ds values or all contigs analysed for gene-exprssion) with the "classic" method and Fisher's exact test.
We used Mann-Whitney u-tests to test the influence of factors on dn/ds values, when multiple contrasts

between groups (facotrs) were investigated we used Nemenyi-Damico-Wolfe-Dunn tests. For overrepresentation of one group (factor) in other groups (factors) we used Fisher's exact test.

General coding methods

The bulk of analysis (unless otherwise cited) presented in this paper was carried out in `R` [51] using custom scripts. We used a method provided in the R-packages `Sweave` [52] and `Weaver` [53] for "reproducible research" combining `R` and LaTeXcode in a single file. All intermediate data files needed to compile the present manuscript from data-sources are provided upon request. For visualisation we used the R-packages `ggplot2` [54] and `VennDiagram` [55].

Results

Sampling A. crassus

One female worm and one male worm were sampled from an aquaculture with height infection loads in Taiwan. An additional female worm was sampled from a stream with low infection pressure adjacent to the aquaculture. All these worms were parasitising endemic *An. japonica*. A female worm and pool of L2 larval stages were sampled from *An. anguilla* in the river Rhine, one female worm from a lake in Poland. All adult worms were filled with large amounts of host-blood, therefore we anticipated abundant host-contamination in sequencing data and decided to sequence a liver sample of an uninfected *An. japonica* for screening.

Sequencing, trimming and pre-assembly screening

A total of 756363 raw sequencing reads were generated for *A. crassus* (Table 1). These were trimmed for base call quality, and filtered by length to give 585949 high-quality reads (spanning 169863104 bases). In the eel dataset from 159370 raw reads 135072 were assembled after basic quality screening.
We then screened the *A. crassus* reads for contamination by host (30071 reads matched previously sequenced eel genes or our own *An. japonica* 454 transcriptome, which had been assembled into 10639 mRNA contigs. 181783 reads matched large or small subunit nuclear or mitochondrial ribosomal RNA sequences of *A. crassus* (Table 1). In addition to fish mRNAs, we identified (and removed) 5286 reads in the library derived from the L2 nematodes that had significant similarity to cercozoan (likely parasite) ribosomal RNA genes (Table 1).

Assembly

We assembled the remaining 353055 reads (spanning 100491819 bases) using the combined assembler strategy [21] and Roche 454 GSassembler (version 2.6) and MIRA (version 3.21) [25]. From this we derived 13851 contigs that were supported by both assembly algorithms, 3745 contigs only supported by one of the assembly algorithms and 22591 singletons that were not assembled by either approach (Table 2). When scored by matches to known genes, the contigs supported by both assemblers are of the highest credibility, and this set is thus termed the high credibility assembly (highCA). Those with evidence from only one assembler and the singletons are of lower credibility (lowCA). These datasets are the most parsimonious (having the smallest size) for their quality (covering the largest amount of sequence in reference transcriptomes). In the highCA parsimony and low redundancy is prioritized, while in the complete assembly (highCA plus lowCA) completeness is prioritized. The 40187 sequences (contig consensuses and singletons) in the complete assembly are referred to below as tentatively unique genes (TUGs).

We screened the complete assembly for residual host contamination, and identified 3441 TUGs that had higher, significant similarity to eel (and chordate) sequences (our and publicly available 454 ESTs and EMBLBank Chordata proteins) than to nematode sequences [28].

Given our prior identification of cercozoan ribosomal RNAs, we also screened the complete assembly for contamination with other transcriptomes.

1153 TUGs were found mapping to Eukaryota outside of the kingdoms Metazoa, Fungi and Viridiplantae. These hits included a wide range of Protists ranging from Apicomplexa (mainly Sarcocystidae, 28 hits and Cryptosporidiidae 10 hits) over Bacillariophyta (diatoms, mainly Phaeodactylaceae, 41 hits) and Phaeophyceae (brown algae, mainly Ectocarpaceae, 180 hits) and Stramenopiles (Albuginaceae, 63 hits) to Kinetoplasitda (Trypanosomatidae, 26 hits) and Heterolobosea (Vahlkampfiidae, 38 hits).

Additionally we found 298 TUGs with hits to fungi (e.g Ajellomycetaceae, 53 hits) and 585 TUGs with hits to plants.

Hits outside the Eukaryota were mainly to Bacteria (825 hits) and within those mostly to members of the Proteobacteria (484 hits). No hits were found to Wolbachia or related Bacteria known as symbionts of nematodes and arthropods. 9 TUGs were hitting sequence from Viruses and 8 from Archaea.

We excluded all TUGs with best hits outside Metazoa and our assembly thus has 32525 TUGs, spanning 154084 bases (of which 11372 are highCA-derived, and span 49741 bases) that are likely to derive from of *A. crassus*.

Protein prediction

For 32418 TUGs a protein was predicted using prot4EST [29] (Table 2). The full open reading frame was obtained in 353 TUGs, while while for 2683 the 5' end and for 8283 the 3' end was complete. In 13383 TUGs the corrected sequence with the imputed ORF was slightly changed compared to the raw sequence.

Annotation

We obtained basic annotations with orthologous sequences from *C. elegans* for 9556 TUGs, from *B. malayi* for 9664 TUGs, from nempep [27, 28] for 11620 TUGs and with uniprot proteins for 11115 TUGs.
We used annot8r [32] to assign gene ontology (GO) terms for 6511 TUGs, Enzyme Commission (EC) numbers for 2460 TUGs and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations for 3846 TUGs (Table 2). Additionally 5125 highCA derived contigs were annotated with GO terms through `InterProScan` [34]. Nearly one third (6989) of the *A. crassus* TUGs were annotated with at least one identifier, and 1831 had GO, EC and KEGG annotations (Figure 1).
We compared our *A. crassus* GO annotations for high-level GO-slim terms to the annotations (obtained the same way) for the complete proteome of the filarial nematode *B. malayi* and the complete proteome of *C. elegans* (Figure 2).
Correlation shows the occurrence of terms for the partial transcriptome of *A. crassus* to be more similar to the proteome of *B. malayi* (0.95; Spearman correlation coefficient) than to the proteome of *C. elegans* (0.9). Also the tow model-nematode compared to each other (0.91) are less similar in the occurrence of terms than the two parasites.
We inferred presence of signal peptide cleavage sites in the predicted protein sequence using `SignalP` [33]. We predicted 920 signal peptide cleavage sites and 65 signal peptides with a transmembrane signature. Again these predictions are more similar to predictions using the same methods for the proteome *B. malayi* (742 signal peptide cleavage sites and 41 with transmembrane anchor) than for the proteome of *C. elegans* (4273 signal peptide cleavage sites and 154 with transmembrane anchor).
We inferred the presence of a lethal rnai phenotype in the orthologous annotation of *C. elegans*. For 259 TUGs a non-lethal phenotype was inferred, for 6029 TUGs a lethal phenotype.

Evolutionary conservation

A. crassus TUGs were classified as conserved, conserved in Metazoa, conserved in Nematoda, conserved in Spirurina or novel to *A. crassus* by comparing them to public databases and using two `BLAST` bit-score

cutoffs to define relatedness (Table 3).

Roughly a third and a quarter of the higCA derived contigs were categorized as conserved across kingdoms at a bitscore threshold of 50 and 80, respectively. Roughly half or 3/5 of the these contigs were identified as novel in *A. crassus*.

The remaining higCA contigs spread across intermediate relatedness-levels. More sequences were categorised as novel at the phylum level (Nematoda) compared to kingdom and clade III level and the number of contigs at intermediate relatedness-levels was roughly consistent for the two bitscore thresholds. The latter points about intermediate conservation levels were also true, when all TUGs were analysed. The numbers of TUGs categorised at these intermediate levels roughly doubled. In contrast, the proportion of additional conserved lowCA TUGs is small compared to additional TUGs categorised as novel in *A. crassus*, mirroring the higher amount of erroneous sequence.

Proteins predicted to be novel to Nematoda and novel in *A. crassus* were significantly enriched in signal peptide annotation compared to conserved proteins, proteins novel in Metazoa and novel in clade III (Fisher's exact test $p<0.001$; Figure 3).

The proportion of lethal rnai phenotypes was significantly higher for orthologs of conserved TUGs (97.23%) than for orthologs of TUGs not conserved (94.59%) across kingdoms ($p<0.001$, Fisher's exact test).

Identification of single nucleotide polymorphisms

We called single nucleotide polymorphisms (SNPs) on the 1100522 bases of the TUGs that had coverage of more then 8-fold available using `VARScan` [39]. We excluded SNPs predicted to have more than 2 alleles or that mapped to an undetermined (N) base in the reference, and retained 10496 SNPs. The ratio of transitions (ti; 6908) to transversion (tv; 3588) in this set was 1.93. Using the prot4EST predictions and the corrected sequences, 7189 of the SNPs were predicted to be inside an ORF, with 2322 at codon first positions, 1832 at second positions and 3035 at third positions. As expected ti/tv inside ORFs (2.39) was higher than outside ORFs (1.25). The ratio of synonymous polymorphisms per synonymous site to non-synonymous polymorphisms per non-synonymous site (dn/ds) was 0.42. We filtered these SNPs to exclude those that might be associated with analytical bias. As Roche 454 sequences have well-known systematic errors associated with homopolymeric nucleotide sequences [56], we analysed the effect of exclusion of SNPs in, or close to, homopolymer regions. We observed changes in ti/tv and in dn/ds when SNPs were discarded using different size thresholds for homopolymer runs and proximity thresholds (see Figure 4). Based on this we decided to exclude SNPs with a homopolymer-run as long as or longer than 4

bases inside a window of 11 bases (5 to bases to the right, 5 to the left) around the SNP. We also observed a relationship between TUG dn/ds and TUG coverage, associated with the presence of sites with low abundance minority alleles (less than 7% of the allele calls), suggesting that some of these may be errors. Removing low abundance minority allele SNPs from the set removed this effect (Figure 5). Our filtered SNP dataset includes 5113 SNPs. We retained 4.65 SNPs per kb of contig sequence, with 8.36 synonymous SNPs per 1000 synonymous bases and 2.4 non-synonymous SNPs per 1000 non-synonymous bases. A mean dn/ds of 0.225 was calculated for the 763 TUGs (763 highCA-derived contigs) containing at least one synonymous SNP.

Polymorphisms associated with biological processes

We consolidated our annotation and polymorphism analyses by examining correlations between nonsynonymous variability and particular classifications.

Signal peptide containing proteins have been shown to have higher rates of evolution than cytosolic proteins in a number of nematode species. In *A. crassus*, TUGs predicted to contain signal peptide cleavage sites in SignalP showed non-significant a trend towards higher dn/ds values than TUGs without signal peptide cleavage sites ($p = 0.184$; two sided Mann-Whitney-test; Figure 6).

Positive selection can be inferred from dn/ds analyses, and we defined TUGs with a dn/ds higher than 0.5 as positively selected. We identified over-represented GO ontology terms associated with these putatively positively selected genes (Table 4; and additional figure 1). Within the molecular function category, "peptidase activity" was the most significantly overrepresented term and had twelve TUGs supporting the overrepresentation. The highlighted twelve peptidases annotated with eleven unique orthologs in *C. elegans* and *B. malayi*.

Other overrepresented terms abundant over categories pointed to subunits of the respiratory chain e.g. "heme-copper terminal oxidase activity" and "cytochrome-c oxidase activity" in molecular function and "mitochondrion" in cellular compartment (see Table 6 and additional figure 1).

At both bitscore thresholds contigs novel in clade III and novel in *A. crassus* had a significantly higher dn/ds than other contigs (novel.in.metazoa - novel.in.Ac, 0.005 and 0.015; novel.in.nematoda - novel.in.Ac, 0.005 and 0.002; novel.in.nematoda - novel.in.clade3, 0.207 and 0.045; comparison, p-value from bitscore of 50 and p-value from bitscore of 80, Nemenyi-Damico-Wolfe-Dunn test, given only for significant comparisons).

Orthologs of *C. elegans* transcripts with lethal rnai phenotype are expected to evolve under stronger

selective constraints. Indeed the values of dn/ds showed a non-significant trend towards lower values in TUGs with orthologs with a lethal phenotype compared to a non-lethal phenotypes (p=0.829, two-sided U-test).

SNP markers for single worms

We used `Samtools` [38] and `Vcftools` [41] to call genotypes in single worms (adult sequencing libraries). This resulted in 199 informative sites in 152 contigs, where two alleles were found in at least one assured genotype at least in one of the worms.

Internal relatedness [43], homozygosity by loci [44] and standardised heterozygosity [45] were all highlighting the Taiwanese worm from the wild population (sample T1) as the most and the European worm from Poland (sample E2) as the least heterozygous individual. The other worms had intermediate values between these two extremes.

We confirmed the genome-wide significance of these estimates using heterozygosity-heterozygosity correlation [42]. These tests confirmed the representativeness of the 199 SNP-markers for the whole genome in population genetic studies ($\mu = 0.78$, ci_l=0.444; $\mu = 0.86$ and $ci_l = 0.596$; $\mu = 0.87$ and ci_l= 0.632; mean and lower bound of 95% confidence intervals from 1000 bootstrap replicates for internal relatedness, homozygosity by loci and standardised heterozygosity). Using a higher number of genotyped individuals these markers would allow to asses the amount of inbreeding in populations of *A. crassus.*.

Differential expression

```
tGO_SEX_EXP_MF_classic_10_all  --- no of nodes:  23
tGO_SEX_EXP_BP_classic_10_all  --- no of nodes:  53
tGO_SEX_EXP_CC_classic_10_all  --- no of nodes:  30

tGO_EEL_EXP_MF_classic_10_all  --- no of nodes:  13
tGO_EEL_EXP_BP_classic_10_all  --- no of nodes:  33
tGO_EEL_EXP_CC_classic_10_all  --- no of nodes:  34
```

We also analysed gene-expression inferred from mapping. Of the 353055 reads 252388 (71.49%) mapped uniquely (with their best hit) to the fullest assembly (including the all assembled contigs as a "filter" later removing screened out sequences for analysis). The number of reads mapping is given for each library Table 1, to get unbiased estimates of expression we removed also all contigs with a coverage lower than 32

reads overall and thus analysed 658 contigs.

54 contigs showed an expression predominantly in the male library, 56 contigs in the female library. 56 contigs were primarily expressed in the libraries from Taiwan, 22 contigs in the European library.

Overrepresentation of of GO-terms differentially expressed between the male and female libraries highlighted especially ribosomal proteins oxidoreductases and collagen processing enzymes as enriched (Table 6 a; additional figures 1). Ribosomal proteins were all overexpressed in the male library, oxidoreductases and collagen processing enzymes were overexpressed female libraries.

Overrepresentation of of GO-terms differentially expressed between libraries from woms of European and Asian origin highlighted catalytic activity especially related to metabolism (Table 6 b; additional Figure 3). Acyltransferase contigs were all upregulated in the European libraries. However, the expression patterns for other contigs connected to metabolsim did not show concerted up or down-regulation (eg. for "steroid biosynthetic process" 2 contigs were downregulated in the European library, 3 contigs upregulated).

Enrichment of signal-positives was not found in any category of overexpressed genes. Differntially expressed genes also showed no pattern of enrichement in conservation categories and no enrichment of *C. elegans* orthologs with lethal/non-lethal rnai-phenotypes.

Significantly elevated dn/ds was found for contigs differentially expressed according to worm-origin (Fisher's exact test p=0.005; also both up- or downregulated were significant). Contigs overexpressed in the female libraries showed elevated levels of dn/ds (Fisher's exact test p=0.035). In contrast male overexpressed genes showed decreased levels of dn/ds (Fisher's exact test p=0.015).

Discussion

We have generated a de novo transcriptome for *A. crassus* an important invasive parasite that threatens wild stocks of the European eel *An. anguilla*. These data enable a broad spectrum of molecular research on this ecologically and economically important parasite. As *A. crassus* lives in close association with its host, we have used exhaustive filtering to attempt to remove all host-derived, and host-associated organism-derived contamination from the data. To do this we have also generated a transcriptome dataset from the definitive host *An. japonica*. The non-nematode, non-eel data identified, particularly in the L2 sample, showed highest identity to flagellate protists, which may have been parasitising the eel (or the nematode). Encapsulated objects observed in eel swim bladder walls [14] could be due solely to immune attrition of *A. crassus* larvae or to other coinfections.

A second examination of sequence origin was performed after assembly, employing higher stringency

cutoffs. Similar taxonomic screening was used in a garter snake transcriptome project [57], and an analysis of lake sturgeon tested and rejected hypotheses of horizontal gene-transfer when xenobiont sequences were identified [58]. A custom pipeline for transcriptome assembly from pyrosequencing reads [59] proposed the use of EST3 [60] to infer sequence origin based simply on nucleotide frequency. We were not able to use this approach successfully, probably due to the fact that xenobiont sequences in our data set derive from multiple sources with different GC content and codon usage.

Compared to other NGS transcriptome sequencing projects [61], the combined assembly approach generated a smaller number of contigs that had lower redundancy and higher completeness. Projects using the mira assembler often report substantially greater numbers of contigs for datasets of similar size (see e.g. [62]), comparable to the mira sub-assembly in our approach. The use of oligo(dT) to capture mRNAs probably explains the bias towards 3' end completeness and a relative lack of true initiation codons in our protein prediction. This bias is near-ubiquitous in deep transcriptome sequencing projects (e.g. [63]).

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We were able to obtain high-quality annotations for a large set of TUGs: For 40% of the complete assembly and 60% of our highCA assembly `BLAST`-based annotations could be obtained. 45% of the contigs in the highCA assembly were additionally decorated with domain-based annotations through `InterProScan` [34]. Comparison with complete protein sequence from the genomes of *B. malayi* and *C. elegans* showed a remarkable degree of agreement regarding the occurrence of terms in the two parasitic worms. This agreement was higher than with the free living nematode *C. elegans* and even the two genome-sequencing-derived proteomes showed less agreement with each other than the filarial parasite with our dataset. This implies that our transcriptome is truly a representative partial genome [64] of a parasitic nematode.

Analysis of conservation identified more sequence novel in Nematode than in the eukaryote kingdom or in clade III this is in agreement with prevalence of genic novelty in the Nematoda [65]. Furthermore the basal position of *A. crassus* in clade III could be leading to most novelty in the clade not being shared with *A. crassus*.

TUGs predicted to be novel in the phylum Nematoda and novel to *A. crassus* contained the highest proportion of signal-positives. This confirms observations made in a study on *Nippostrongylus brasiliensis* [66], where signal positives were reported as less conserved. Interestingly enrichment of signal sequence bearing TUGs in our dataset was constrained to sequences novel in nematodes and *A. crassus*

(i.e. not to the level of clade III). This may be explained, whit two different hypotheses involving the basal position of *A. crassus*: First the signal positives shared with all nematodes could be conserved molecules not excreted by parasites. A different class of secreted/excreted molecules with prominent role in host parasite interactions would not have arisen early in the evolution of parasitism in clade III - or be too fast-evolving - and thus be detected as specific to deeper sub-clades (i.e. to *A. crassus* in our dataset). A second explanation would be, that orthologs of excreted parasite-specific genes could be among those shared with other nematodes and the fewer shared with clade III implying a predisposition to parasitism outside of the Spirurina or even the convergent evolution of secreted molecules in other parasitic nematodes. However analysis of dn/ds (see below) across conservation categories favours the first hypothesis, as it identifies a higher amount of positive selection in TUGs novel to clade III and *A. crassus* than to nematodes.

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We generated transcriptome data from multiple *A. crassus* of Taiwanese and European origin, and identified SNPs both within and between populations. Screening of SNPs in or adjacent to homopolymer regions improved overall measurements of SNP quality. The ratio of transitions to transversions (ti/tv) increased. Such an increase is explained by the removal of "noise" associated with common homopolymer errors [56]. The value of 1.92530657748049 (1.25 outside, 2.39 inside ORFs) is in good agreement with the overall ti/tv of humans (2.16 [67]) or *Drosophila* (2.07 [68]). The ratio of non-synonymous SNPs per non-synonymous site to synonymous SNPs per synonymous site (dn/ds) decreased with removal of SNPs adjacent to homopolymer regions from 0.42 to 0.225 after full screening. The most plausible explanation is the removal of error, as unbiased error would lead to a dn/ds of 1. While dn/ds is not unproblematic to interpret within populations [69], the assumption of negative (purifying) selection on most protein-coding genes makes lower mean values seem more plausible. We used a threshold value for the minority allele of 7% for exclusion of SNPs, based on an estimate that approximately 10 haploid equivalents were sampled (5 individual worms plus an negligible contribution from L2 larvae in the L2 library and within the female adult worms). The benefit of this screening was mainly a reduction of non-synonymous SNPs in high coverage contigs, and a removal of the dependence of dn/ds on coverage. Working with an estimate of dn/ds independent of coverage, efforts to control for sampling biased by depth (i.e. coverage; see [70] and [61]) could be avoided.

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Also in comparison with published intra-species values of dn/ds our final estimate of seems plausible: in transcripts from the female reproductive tract of *Drosophila* dn/ds was 0.15 [71] and 0.21 in the male

reproductive tract [72] (although for ESTs specific to the male accessory gland were shown to have a higher dn/ds of 0.47). A pyrosequencing study in the parasitic nematode *Ancylostoma canium* [73] reported dn/ds of 0.3.

When the whole of coding sequences are studied, of which only a small subset of sites can be under diversifying selection, dn/ds of 0.5 has been suggested as threshold for assuming diversifying selection [71] instead of the classical threshold of 1 [74]. The use of this threshold for positive selection led to the identification of over-represented of GO-term highlighting very interesting transcripts:

Twelve peptidases under positive selection (from 43 with a dn/ds obtained) meant an enrichment in the category. All twelve have different orthologs in *B. malayi* and *C. elgans* and are conserved across kingdoms. Despite their conservation peptidases are thought to have acquired new and prominent roles in host-parasite interaction compared to free living organisms: In *A. crassus* a trypsin-like proteinase has been identified thought to be utilised by the tissue-dwelling L3 stage to penetrate host tissue and an aspartyl proteinase thought to be a digestive enzyme in adults [75]. The twelve proteinases under positive selection could be the targets of the adaptive immunity developed against *A. crassus* [13, 76], which is often only elicited against subtypes of larvae [77].

Genotyping of individual worms identified a set of 199 SNPs with highest credibility and a high information content for population-genetic studies. Levels of genome-wide heterozygosity found for the 5 adult worms examined in our study are in agreement with microsattelite data [78] showing reduced heterozygosity in European populations of *A. crassus*.

We employed methods to developed for the comparison of cDNA-libraries to make inferrence about possible differental gene-expression according to experimental groups (origin of sequencing-libraries) [47]. Such approaches are widely used with pyrosequencing-data (e.g. [73]). For the statistically valid comparison of conditions however, the unit of replication whould be the individual library and approaches respecting this fact would be desirable. However we were not able to use the R-packages DESeq [79] or edgeR [80] developed for count data from deep sequencing (but more targeted towards rna-seq on the solexa-plattform) as both repetition and throughput of our present experiment were too low. As a result the differentially expressed genes are by no means significant for the investigated conditions, but just for the specific cDNA-libraries. With these reservations we identified genes differentially expressed between libraries prepared form worms of different sex and worms from different origin.

Genes over-expressed in male *A. crassus* comprise major sperm proteins well known for their high expression in nematode sperm [81]. A surprise was the overexpression of ribosomal proteins in the male

library.

That collagen processing enzymes are overexpressed in female worms, filled with developing embyos and larvae, is in line with a complicated regulation and modulation of collagen in nematode larval development [82].

The overexpression acetyl-CoA acetyltransferase in European woms are interesting expecially because of the role of these enzymes in fatty-acid β-oxidation in peroxisomes and mitochondria [83]. Together with a change in steroid metabolism and the enrichment of mitochondrially localized enzymes these are suggestive of changes in energy metabolism of *A. crassus* form different origins. Possible explanations would include a change to more or less aerobic processes in worms in Europe due to their bigger size and/or increased availablilty of nutrients.

Contigs overexpressed in the female libraries showed elevated levels of dn/ds but genes overexpressed in males decreased levels of dn/ds. The first finding is unexpected, as overexpressed in female libraries will also contain contigs related to larval development (such as the collagen modifying enzymes discussed above), these larval transcripts in turn are expected to be under purifying selection because of pleiotropic effects of genes in early development [84]. Also the second finding is in slight contrast to pubished results for male specific traits and transcripts are often showning hallmarks of positive selection [72, 85]. . In *Ancylostoma caninum* however, female-specific transcipts showed an enrichment fof "parasitism genes" [73] and a possible expantion would be a similar enrichment of positively selected parasitism related in our dataset. For males the decreased dn/ds can be explained by the by the high number of ribosomal proteins, which are all show very low levels of dn/ds (that these proteins are found differentially expressed remaines puzzling though), while single transcripts e.g. major sperm protein (exressed in the male library only) showed elevated dn/ds but did not level the overall effect. But this also has a positve aspect: it is unlikely that correlation of differential expression with positive selection results from mapping artefacts, as all the ribosomal proteins identified overexpressed in males have very low dn/ds.

Genes differentiall expressed according to worm-origin (in either direction) showed significantly elevated levels of dn/ds. This is interpretable as a correlation between sequence evolution and phenotypic modification in different host-environments or even correation between sequence evolution and evolution of gene-expression. Thus, whether expression of these genes is modified in different hosts or evolved rapidly in a contemporary divergence between European and Asian populations of *A. crassus*, is in the center of a future research programm building on the reference transcriptome presented here. For such an annalysis it is important to disentangle the influence of the host and the nematode population in a co-inoculation

experiment. Such a project will also use the individual worm as the level of replication for "conditions" (that is, worm-population and host-species) to allow rigid hypothesis testing. Based on the pilot evalutaion presented here differences in these factors are expected overlap with differences in male vs. female worms and the careful cross-examination of the above factors with worm-sex is adviced.
Population genetic approaches using the SNP-markers presented here directly or populations genomic approaches choosing to use the SNPS found here as gold-standard in comparison with higher throughput technology, constitute another field of future research on *A. crassus*. The maintenance or loss of variation in European populations in or close to genes under general positive selection will be of major interest in such projects.

Conclusions

The *A. crassus* transcriptome provides a basis of molecular research on this ecologically important species. It further allows insight in the evolution of parasitism complementing the catalogue of available transcriptomic data with a member of the Spirurina phylogenetically distant to so far sequenced parasites in this clade. Differences in energy metabolism between European and Asian *A. crassus* constitute a candidate phenotype relevant for phenotypic modification or contemporary divergent evolution as well as for the long term evolution of parasitism.

Competing interests

The authors declare no competing interests.

Authors contributions

EGH and MB conceived and designed the experiments. EGH carried out bioinformatic analyses. SB assisted in bioinformatic analyses. AM prepared sequencing libraries. HT provided close supervision throughout. EGH and MB interpreted results and prepared the manuscript. All authors have read and approved the final manuscript.

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Figures

Figure 1 - Number of contigs annotated with different methods

Number of annotations obtained for Gene Ontology (GO), Enzyme Commission (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms through `Annot8r` [32] for all TUGs (a) and for higCA derived contigs (b). The latter includes additional domain-based annotations obtained with `InterProScan` [34].

Figure 2 - Comparing high level GO-slim annotations

For Gene Ontology (GO) categories molecular function, cellular compartment and biological process the number of terms in high level GO-slim categories is given as obtained through `Annot8r` [32].

Figure 3 - Enrichment of Signal-positives for categories of evolutionary conservations

Proportions of `SignalP`-predictions for each category of evolutionary conservation. Generally - across bit-score thresholds - TUGS novel in nematodes and in *A. crassus* have the highest proportion of signal-positives.

Figure 4 - Changes in ti/tv and dn/ds due to exclusion of homopolymer-runs

When SNPs in or adjacent to homopolymeric regions are removed changes in ti/tv and dn/ds are observed: As the overall number of SNPs is reduced both ratios change to more plausible values. Note the reversed axis for dn/ds to plot these lower values to the right. For homopolymer length > 3 a linear trend for the total number of SNPs and the two measurements is observed. A width of 11 for the screening window provides most plausible values (suggesting specificity) while still incorporating a high number of SNPs (sensitivity).

Figure 5 - SNP calling and SNP categories

Overabundance of SNPs at (a) codon-position two and of (c) non-synonymous SNPs for low percentages of the minority allele. (b) Significant positive correlation of coverage and dn/ds before removing these SNPs at a threshold of 7% ($p < 0.001$, $R^2 = 0.017$) and (d) afterwards ($R^2 <$0.001, $p =$0.195).

Figure 6 - Positive selection and evolutionary conservation

Box-plots for dn/ds in TUGs according to different categories of evolutionary conservation. Significant comparisons are novel.in.metazoa - novel.in.Ac (0.005 and 0.015), novel.in.nematoda - novel.in.Ac (0.005

and 0.002), novel.in.nematoda - novel.in.clade3 (0.207 and 0.045; p-value for bitscore of 50 and 80, Nemenyi-Damico-Wolfe-Dunn test).

Tables

Table 1 - Sampling, trimming and pre-assembly screening, library statistics

For libraries two sequencing libraries from European eels (E1 and E2) one form L2-larvae (L2), one from male (M) and two from Eels in Taiwan (T1 and T2) the following statistics are given. life.st = lifecycle stage: f for female m for male. source.p = source population: R for Rhine, P for Poland, C for cultured, W for wild. raw.reads = raw number of sequencing reads obtained. lowqal = number of reads discarded due to low quality or length in *Seqclean* [23]. AcrRNA = number of reads hitting *A. crassus*-rRNA (screened). eelmRNA = number of reads hitting eel transcriptome-sequences (screened). eelrRNA = number of reads hitting eel-rRNA genes (screened). Cercozoa = number of reads hitting cercozoan rRNA (screened). valid = number of reads valid after screening (assembled). valid.span = number of bases valid (assembled). mapping.unique = number of reads mapping uniquely to the assembly. mapping.Ac = number of reads mapping to the part of the assembly considered *A. crassus* origin (see post-assembly screening). mapping.MN = number of reads mapping to the highCA-derived part of the assembly (and also *A. crassus* origin). over.32 = number of reads mapping to contigs with overall coverage of more than 32 reads (considered in gene-expression analysis).

library	E1	E2	M	T1	T2
life.st	adult f	adult f	adult m	adult f	adult f
source.p	Europe R	Europe P	Asia C	Asia C	Asia W
raw.reads	209325	111746	106726	99482	116366
lowqal	92744	10903	15484	7947	27683
AcrRNA	76403	11213	31351	24929	7233
eelmRNA	4835	3613	1187	7475	11741
eelrRNA	13112	69	418	514	38
Cercozoa	0	0	0	0	0
valid	22231	85948	58286	58617	69671
valid.span	7167338	24046225	17424408	14443123	20749177
mapping.unique	12023	65398	36782	42529	55966
mapping.Ac	8359	61073	31673	37306	50445
mapping.MN	5883	48009	18998	28970	41963
over.32	3528	34054	10456	21366	22853

Table 2 - assembly classification and contig statistics

Summary statistics for contigs from different assembly-categories given in columns as highCA = high credibility assembly; lowCA = low credibility assembly, combined = complete assembly.
Rows indicate summary statistics: total.contigs = numbers of total contigs, fish.contigs = number of contigs hitting eel-mRNA or Chordata in NCBI-nr or NCBI-nt (screened out), xeno.contigs = number of contigs with best hit (NCBI-nr and NCBI-nt) to non-eukaryote (screened out), remaining.contigs = number

of contigs remaining after this screening, remaining.span = total length of remaining contigs, non.u.cov = non-unique mean base coverage of contigs, cov = unique mean base coverage of contigs, p4e."X" = number protein predictions derived in p4e, where "X" describes the method of prediction (see Methods), full.3p = number of contigs complete at 3', full.5p = number of contigs complete at 5', GO = number of contigs with GO-annotation, KEGG = number of contigs with KEGG-annotation, EC = number of contigs with EC-annotation, nem.blast = number of contigs with `BLAST`-hit to nematode in nr, any.blast = number of contigs with `BLAST`-hit to non-nematode (eukaryote non chordate) sequence in NCBI-nr.

	lowCA	highCA	combined
total.contigs	26336	13851	40187
rRNA.contigs	829	59	888
fish.contigs	2419	1022	3441
xeno.contigs	1935	1398	3333
remaining.contigs	21153	11372	32525
remaining.span	8095986	7971550	16067536
non.u.cov	14.665	10.979	12.840
cov	2.443	6.838	4.624
p4e.BLAST-similarity	4357	5664	10021
p4e.ESTScan	8324	3597	11921
p4e.LongestORF	8352	2085	10437
p4e.no-prediction	93	14	107
full.3p	5909	2714	8623
full.5p	1484	1270	2754
full.l	104	185	289
GO	2636	3875	6511
EC	967	1493	2460
KEGG	1609	2237	3846
IPR	0	7557	7557
nem.blast	4869	5821	10690
any.blast	5107	6008	11115

Table 3 - Evolutionary conservation and novelty

The kingdom Metazoa (novel.in.metazoa), the phylum Nematoda(novel.in.nematoda) and clade III (Spirurina; novel.in.spirurina) were assessed for occurrences of `BLAST`-hits at two different bitscore thresholds (50 = bit.50 and 80 = bit.80). TUGs without any hit at a given threshold were categorized as novel in *A. crssus* (novel.in.Ac). Both novelty and conservation can be derived from this (numbers for conservation would be the cumulative sum of lower-level novelty).

	conserved	novel.in.metazoa	novel.in.nematoda	novel.in.clade3	novel.in.Ac
bit.50.all	5604	1715	2173	1485	21548
bit.80.all	3506	1383	2015	1525	24096
bit.50.highCA	3479	876	1010	601	5406
bit.80.highCA	2457	833	1084	716	6282

Table 4 - Over-representation of GO-terms in positively selected

Significantly ($p<0.05$) over-represented GO-terms in contigs putatively under positive selection. Horizontal lines separate categories of the GO-ontology. First category is molecular function, second biological process, last cellular compartment. P-values (p.value) for over-representation are given along with the number of positively selected contigs (Significant; $dn/ds > 0.5$) and the number of contigs with this annotation for which a dn/ds was obtained (Annotated) and the description of the GO-term (Term). For a graph of incuced GO-terms see also addional figure 1.

GO.ID	Term	Annotated	Significant	Expected	p.value
GO:0008233	peptidase activity	43	12	5.25	0.0028
GO:0015179	L-amino acid transmembrane transporter activity	2	2	0.24	0.0147
GO:0016787	hydrolase activity	110	20	13.42	0.0256
GO:0043021	ribonucleoprotein binding	6	3	0.73	0.0264
GO:0005102	receptor binding	26	7	3.17	0.0286
GO:0046982	protein heterodimerization activity	16	5	1.95	0.0346
GO:0004129	cytochrome-c oxidase activity	3	2	0.37	0.0405
GO:0004540	ribonuclease activity	3	2	0.37	0.0405
GO:0005275	amine transmembrane transporter activity	3	2	0.37	0.0405
GO:0005342	organic acid transmembrane transporter activity	3	2	0.37	0.0405
GO:0015002	heme-copper terminal oxidase activity	3	2	0.37	0.0405
GO:0015171	amino acid transmembrane transporter activity	3	2	0.37	0.0405
GO:0016675	oxidoreductase activity, acting on a heme group of donors	3	2	0.37	0.0405

GO:0016676	oxidoreductase activity, acting on a heme group of donors, oxygen as acceptor	3	2	0.37	0.0405
GO:0046943	carboxylic acid transmembrane transporter activity	3	2	0.37	0.0405
GO:0047035	testosterone dehydrogenase (NAD+) activity	3	2	0.37	0.0405
GO:0015077	monovalent inorganic cation transmembrane transporter activity	12	4	1.46	0.0468
GO:0070011	peptidase activity, acting on L-amino acid peptides	35	8	4.27	0.0496
GO:0009081	branched chain family amino acid metabolic process	3	3	0.36	0.0017
GO:0009083	branched chain family amino acid catabolic process	3	3	0.36	0.0017
GO:0042594	response to starvation	15	6	1.81	0.0051
GO:0006914	autophagy	12	5	1.45	0.0089
GO:0006520	cellular amino acid metabolic process	44	11	5.32	0.0101
GO:0007281	germ cell development	17	6	2.05	0.0104
GO:0090068	positive regulation of cell cycle process	17	6	2.05	0.0104
GO:0009308	amine metabolic process	57	13	6.89	0.0116
GO:0051325	interphase	23	7	2.78	0.0137
GO:0051329	interphase of mitotic cell cycle	23	7	2.78	0.0137
GO:0010564	regulation of cell cycle process	34	9	4.11	0.0138
GO:0051726	regulation of cell cycle	52	12	6.28	0.0140
GO:0009056	catabolic process	149	26	18.01	0.0144
GO:0005997	xylulose metabolic process	2	2	0.24	0.0144
GO:0006739	NADP metabolic process	2	2	0.24	0.0144
GO:0009744	response to sucrose stimulus	2	2	0.24	0.0144
GO:0010172	embryonic body morphogenesis	2	2	0.24	0.0144

GO:0015807	L-amino acid transport	2	2	0.24	0.0144
GO:0019321	pentose metabolic process	2	2	0.24	0.0144
GO:0034285	response to disaccharide stimulus	2	2	0.24	0.0144
GO:0050885	neuromuscular process controlling balance	2	2	0.24	0.0144
GO:0006915	apoptosis	78	16	9.43	0.0145
GO:0031571	mitotic cell cycle G1/S transition DNA damage checkpoint	14	5	1.69	0.0185
GO:0044106	cellular amine metabolic process	55	12	6.65	0.0221
GO:0009063	cellular amino acid catabolic process	10	4	1.21	0.0232
GO:0000082	G1/S transition of mitotic cell cycle	15	5	1.81	0.0253
GO:0030330	DNA damage response, signal transduction by p53 class mediator	15	5	1.81	0.0253
GO:0031575	mitotic cell cycle G1/S transition checkpoint	15	5	1.81	0.0253
GO:0033238	regulation of cellular amine metabolic process	15	5	1.81	0.0253
GO:0042770	signal transduction in response to DNA damage	15	5	1.81	0.0253
GO:0071779	G1/S transition checkpoint	15	5	1.81	0.0253
GO:0072331	signal transduction by p53 class mediator	15	5	1.81	0.0253
GO:2000045	regulation of G1/S transition of mitotic cell cycle	15	5	1.81	0.0253
GO:0006401	RNA catabolic process	6	3	0.73	0.0258
GO:0010638	positive regulation of organelle organization	6	3	0.73	0.0258
GO:0042981	regulation of apoptosis	64	13	7.73	0.0307
GO:0043067	regulation of programmed cell death	64	13	7.73	0.0307

GO:0009310	amine catabolic process	11	4	1.33	0.0333
GO:0051084	'de novo' posttranslational protein folding	11	4	1.33	0.0333
GO:0008219	cell death	93	17	11.24	0.0363
GO:0016265	death	93	17	11.24	0.0363
GO:0012501	programmed cell death	86	16	10.39	0.0365
GO:0010941	regulation of cell death	66	13	7.98	0.0391
GO:0000393	spliceosomal conformational changes to generate catalytic conformation	3	2	0.36	0.0398
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	3	2	0.36	0.0398
GO:0006865	amino acid transport	3	2	0.36	0.0398
GO:0009313	oligosaccharide catabolic process	3	2	0.36	0.0398
GO:0031023	microtubule organizing center organization	3	2	0.36	0.0398
GO:0045292	nuclear mRNA cis splicing, via spliceosome	3	2	0.36	0.0398
GO:0045840	positive regulation of mitosis	3	2	0.36	0.0398
GO:0051262	protein tetramerization	3	2	0.36	0.0398
GO:0051289	protein homotetramerization	3	2	0.36	0.0398
GO:0051297	centrosome organization	3	2	0.36	0.0398
GO:0051785	positive regulation of nuclear division	3	2	0.36	0.0398
GO:2000242	negative regulation of reproductive process	3	2	0.36	0.0398
GO:0007286	spermatid development	7	3	0.85	0.0413
GO:0009267	cellular response to starvation	7	3	0.85	0.0413
GO:0048515	spermatid differentiation	7	3	0.85	0.0413
GO:0016071	mRNA metabolic process	47	10	5.68	0.0432

GO:0006458	'de novo' protein folding	12	4	1.45	0.0454
GO:0022607	cellular component assembly	103	18	12.45	0.0476
GO:0030532	small nuclear ribonucleoprotein complex	7	4	0.84	0.005
GO:0005682	U5 snRNP	2	2	0.24	0.014
GO:0015030	Cajal body	2	2	0.24	0.014
GO:0046540	U4/U6 x U5 tri-snRNP complex	2	2	0.24	0.014
GO:0016607	nuclear speck	6	3	0.72	0.025
GO:0005739	mitochondrion	137	23	16.44	0.033
GO:0005604	basement membrane	3	2	0.36	0.039
GO:0060198	clathrin sculpted vesicle	3	2	0.36	0.039

0.1 Table 5 - Measurements of multi-locus heterozygosity for single worms

Genotyping for a set of 199 SNPs, different measurements were obtained to asses genome-wide heterozygosity. Measurements for relative heterozygosity (rel.het; number of homozygous sites/ number of heterozygous sites), internal relatedness (int.rel; [43]), homozygosity by loci (ho.loci; [44]) and standardized heterozygosity (std.het; [45]) are given. All these measurements are pointing to sample T1 (Taiwanese worm from a wild population) as the most heterozygous and sample E2 (the European worm from Poland) as the least heterozygous individual. Heterozygote-heterozygote correlation [42] confirmed the genome-wide significance of these markers.

	rel.het	int.rel	ho.loci	std.het
T2	0.45	-0.73	0.59	1.00
T1	0.93	-0.95	0.34	1.62
M	0.37	-0.73	0.66	0.84
E1	0.38	-0.83	0.60	0.91
E2	0.18	-0.35	0.82	0.50

0.2 Table 6 - Over-representation of GO-terms differentially expressed

Significantly ($p<0.05$) over-represented GO-terms in contigs differentially expressed between male and female worms (a) or between European and Asian origin (b). Horizontal lines separate categories of the GO-ontology. First category is molecular function, second biological process, last cellular compartment. P-values (p.value) for over-representation are given along with the number of differentially expressed

contigs (Significant) and the number of contigs with this annotation analysed (Annotated) and the description of the GO-term (Term). For a graph of incuced GO-terms see also addional figures 1. a)

GO.ID	Term	Annotated	Significant	Expected	p.value
GO:0005198	structural molecule activity	51	18	8.28	0.00019
GO:0016706	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxyge...	3	3	0.49	0.00407
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxyge...	4	3	0.65	0.01441
GO:0004656	procollagen-proline 4-dioxygenase activity	2	2	0.32	0.02595
GO:0019798	procollagen-proline dioxygenase activity	2	2	0.32	0.02595
GO:0031543	peptidyl-proline dioxygenase activity	2	2	0.32	0.02595
GO:0031545	peptidyl-proline 4-dioxygenase activity	2	2	0.32	0.02595
GO:0034641	cellular nitrogen compound metabolic process	159	37	25.03	0.00020
GO:0048731	system development	146	35	22.98	0.00020
GO:0034621	cellular macromolecular complex subunit organization	73	22	11.49	0.00026
GO:0006807	nitrogen compound metabolic process	162	37	25.50	0.00034
GO:0032774	RNA biosynthetic process	70	21	11.02	0.00043
GO:0071822	protein complex subunit organization	71	21	11.18	0.00055
GO:0043933	macromolecular complex subunit organization	82	23	12.91	0.00063

GO:0000022	mitotic spindle elongation	19	9	2.99	0.00080
GO:0051231	spindle elongation	19	9	2.99	0.00080
GO:0044281	small molecule metabolic process	188	40	29.59	0.00082
GO:0006139	nucleobase-containing compound metabolic process	139	32	21.88	0.00157
GO:0048856	anatomical structure development	188	39	29.59	0.00241
GO:0071841	cellular component organization or biogenesis at cellular level	139	31	21.88	0.00408
GO:0090304	nucleic acid metabolic process	105	25	16.53	0.00546
GO:0071842	cellular component organization at cellular level	135	30	21.25	0.00559
GO:0016070	RNA metabolic process	96	23	15.11	0.00797
GO:0040007	growth	138	30	21.72	0.00847
GO:0050789	regulation of biological process	198	39	31.17	0.00952
GO:0042274	ribosomal small subunit biogenesis	10	5	1.57	0.01084
GO:0009791	post-embryonic development	116	26	18.26	0.01151
GO:0007275	multicellular organismal development	221	42	34.79	0.01156
GO:0022414	reproductive process	105	24	16.53	0.01280
GO:0042157	lipoprotein metabolic process	7	4	1.10	0.01335
GO:0007051	spindle organization	27	9	4.25	0.01435
GO:0007052	mitotic spindle organization	27	9	4.25	0.01435
GO:0040009	regulation of growth rate	62	16	9.76	0.01599
GO:0040010	positive regulation of growth rate	62	16	9.76	0.01599
GO:0018988	molting cycle, protein-based cuticle	23	8	3.62	0.01616
GO:0018996	molting cycle, collagen and cuticulin-based cuticle	23	8	3.62	0.01616

GO:0010467	gene expression	114	25	17.94	0.01935
GO:0042303	molting cycle	24	8	3.78	0.02127
GO:0071840	cellular component organization or biogenesis	171	34	26.92	0.02143
GO:0032501	multicellular organismal process	241	44	37.94	0.02183
GO:0009416	response to light stimulus	8	4	1.26	0.02360
GO:0032502	developmental process	227	42	35.73	0.02409
GO:0008543	fibroblast growth factor receptor signaling pathway	2	2	0.31	0.02437
GO:0018401	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	2	2	0.31	0.02437
GO:0019471	4-hydroxyproline metabolic process	2	2	0.31	0.02437
GO:0019511	peptidyl-proline hydroxylation	2	2	0.31	0.02437
GO:0046887	positive regulation of hormone secretion	2	2	0.31	0.02437
GO:0071570	cement gland development	2	2	0.31	0.02437
GO:0000279	M phase	44	12	6.93	0.02555
GO:0009792	embryo development ending in birth or egg hatching	123	26	19.36	0.02787
GO:0016043	cellular component organization	167	33	26.29	0.02838
GO:0009152	purine ribonucleotide biosynthetic process	5	3	0.79	0.02925
GO:0009260	ribonucleotide biosynthetic process	5	3	0.79	0.02925
GO:0002164	larval development	106	23	16.69	0.03108
GO:0042254	ribosome biogenesis	21	7	3.31	0.03144
GO:0000003	reproduction	137	28	21.56	0.03399
GO:0022613	ribonucleoprotein complex biogenesis	26	8	4.09	0.03482
GO:0065007	biological regulation	217	40	34.16	0.03874
GO:0007010	cytoskeleton organization	57	14	8.97	0.03908
GO:0045927	positive regulation of growth	68	16	10.70	0.03978

GO:0071843	cellular component biogenesis at cellular level	27	8	4.25	0.04344
GO:0048518	positive regulation of biological process	127	26	19.99	0.04357
GO:0034645	cellular macromolecule biosynthetic process	103	22	16.21	0.04358
GO:0000226	microtubule cytoskeleton organization	32	9	5.04	0.04471
GO:0007017	microtubule-based process	32	9	5.04	0.04471
GO:0006364	rRNA processing	18	6	2.83	0.04643
GO:0044267	cellular protein metabolic process	134	27	21.09	0.04769
GO:0002119	nematode larval development	104	22	16.37	0.04876
GO:0009059	macromolecule biosynthetic process	104	22	16.37	0.04876
GO:0030529	ribonucleoprotein complex	62	20	9.84	0.00022
GO:0043228	non-membrane-bounded organelle	115	28	18.25	0.00178
GO:0043232	intracellular non-membrane-bounded organelle	115	28	18.25	0.00178
GO:0044444	cytoplasmic part	258	48	40.95	0.00181
GO:0043227	membrane-bounded organelle	251	47	39.84	0.00274
GO:0043231	intracellular membrane-bounded organelle	251	47	39.84	0.00274
GO:0005829	cytosol	149	33	23.65	0.00306
GO:0031981	nuclear lumen	66	18	10.48	0.00538
GO:0005618	cell wall	17	7	2.70	0.00922
GO:0070013	intracellular organelle lumen	92	22	14.60	0.01115
GO:0043226	organelle	270	48	42.86	0.01309
GO:0043229	intracellular organelle	270	48	42.86	0.01309
GO:0030312	external encapsulating structure	18	7	2.86	0.01324
GO:0044446	intracellular organelle part	193	38	30.63	0.01332
GO:0009536	plastid	27	9	4.29	0.01507
GO:0044422	organelle part	195	38	30.95	0.01703
GO:0043233	organelle lumen	95	22	15.08	0.01721
GO:0022627	cytosolic small ribosomal subunit	15	6	2.38	0.01909

GO:0031974	membrane-enclosed lumen	97	22	15.40	0.02257
GO:0045169	fusome	2	2	0.32	0.02477
GO:0070732	spindle envelope	2	2	0.32	0.02477
GO:0015935	small ribosomal subunit	16	6	2.54	0.02684
GO:0005737	cytoplasm	275	48	43.65	0.02798
GO:0009507	chloroplast	25	8	3.97	0.02868
GO:0005791	rough endoplasmic reticulum	5	3	0.79	0.02991
GO:0005811	lipid particle	30	9	4.76	0.03102
GO:0005773	vacuole	46	12	7.30	0.03833

b)

GO.ID	Term	Annotated	Significant	Expected	p.value
GO:0016408	C-acyltransferase activity	3	3	0.37	0.0018
GO:0016453	C-acetyltransferase activity	3	3	0.37	0.0018
GO:0016407	acetyltransferase activity	4	3	0.50	0.0065
GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups	4	3	0.50	0.0065
GO:0003824	catalytic activity	158	27	19.62	0.0088
GO:0016746	transferase activity, transferring acyl groups	8	4	0.99	0.0099
GO:0001871	pattern binding	2	2	0.25	0.0151
GO:0003682	chromatin binding	2	2	0.25	0.0151
GO:0003985	acetyl-CoA C-acetyltransferase activity	2	2	0.25	0.0151
GO:0003988	acetyl-CoA C-acyltransferase activity	2	2	0.25	0.0151
GO:0008061	chitin binding	2	2	0.25	0.0151
GO:0030247	polysaccharide binding	2	2	0.25	0.0151
GO:0003713	transcription coactivator activity	6	3	0.75	0.0273
GO:0005543	phospholipid binding	6	3	0.75	0.0273
GO:0004090	carbonyl reductase (NADPH) activity	3	2	0.37	0.0417
GO:0008289	lipid binding	12	4	1.49	0.0483
GO:0016853	isomerase activity	12	4	1.49	0.0483

GO:0016126	sterol biosynthetic process	5	4	0.60	0.00083
GO:0048732	gland development	9	5	1.08	0.00173
GO:0016125	sterol metabolic process	6	4	0.72	0.00228
GO:0006694	steroid biosynthetic process	10	5	1.20	0.00316
GO:0006338	chromatin remodeling	4	3	0.48	0.00596
GO:0006695	cholesterol biosynthetic process	4	3	0.48	0.00596
GO:0008203	cholesterol metabolic process	4	3	0.48	0.00596
GO:0044281	small molecule metabolic process	188	30	22.63	0.00748
GO:0008202	steroid metabolic process	12	5	1.44	0.00825
GO:0042180	cellular ketone metabolic process	57	13	6.86	0.00845
GO:0023051	regulation of signaling	28	8	3.37	0.01087
GO:0019219	regulation of nucleobase-containing compound metabolic process	41	10	4.94	0.01412
GO:0001655	urogenital system development	2	2	0.24	0.01416
GO:0001822	kidney development	2	2	0.24	0.01416
GO:0006611	protein export from nucleus	2	2	0.24	0.01416
GO:0007528	neuromuscular junction development	2	2	0.24	0.01416
GO:0009953	dorsal/ventral pattern formation	2	2	0.24	0.01416
GO:0048581	negative regulation of post-embryonic development	2	2	0.24	0.01416
GO:0048741	skeletal muscle fiber development	2	2	0.24	0.01416
GO:0051124	synaptic growth at neuromuscular junction	2	2	0.24	0.01416
GO:0070050	neuron homeostasis	2	2	0.24	0.01416
GO:0072001	renal system development	2	2	0.24	0.01416

GO:0006082	organic acid metabolic process	54	12	6.50	0.01489
GO:0019752	carboxylic acid metabolic process	54	12	6.50	0.01489
GO:0043436	oxoacid metabolic process	54	12	6.50	0.01489
GO:0008152	metabolic process	266	37	32.02	0.01526
GO:0006355	regulation of transcription, DNA-dependent	30	8	3.61	0.01697
GO:0019953	sexual reproduction	44	10	5.30	0.02361
GO:0048747	muscle fiber development	6	3	0.72	0.02503
GO:0051171	regulation of nitrogen compound metabolic process	51	11	6.14	0.02556
GO:0009966	regulation of signal transduction	21	6	2.53	0.02842
GO:0032787	monocarboxylic acid metabolic process	21	6	2.53	0.02842
GO:0051252	regulation of RNA metabolic process	33	8	3.97	0.03036
GO:0048545	response to steroid hormone stimulus	16	5	1.93	0.03141
GO:0065008	regulation of biological quality	81	15	9.75	0.03399
GO:0050794	regulation of cellular process	151	24	18.18	0.03420
GO:0010033	response to organic substance	60	12	7.22	0.03487
GO:0048609	multicellular organismal reproductive process	60	12	7.22	0.03487
GO:0002026	regulation of the force of heart contraction	3	2	0.36	0.03923
GO:0007416	synapse assembly	3	2	0.36	0.03923
GO:0007431	salivary gland development	3	2	0.36	0.03923
GO:0007435	salivary gland morphogenesis	3	2	0.36	0.03923
GO:0007559	histolysis	3	2	0.36	0.03923
GO:0007595	lactation	3	2	0.36	0.03923
GO:0016271	tissue death	3	2	0.36	0.03923
GO:0022612	gland morphogenesis	3	2	0.36	0.03923
GO:0030518	steroid hormone receptor signaling pathway	3	2	0.36	0.03923

GO:0030522	intracellular receptor mediated signaling pathway	3	2	0.36	0.03923
GO:0030879	mammary gland development	3	2	0.36	0.03923
GO:0034612	response to tumor necrosis factor	3	2	0.36	0.03923
GO:0035070	salivary gland histolysis	3	2	0.36	0.03923
GO:0035071	salivary gland cell autophagic cell death	3	2	0.36	0.03923
GO:0035220	wing disc development	3	2	0.36	0.03923
GO:0035272	exocrine system development	3	2	0.36	0.03923
GO:0043628	ncRNA 3'-end processing	3	2	0.36	0.03923
GO:0045540	regulation of cholesterol biosynthetic process	3	2	0.36	0.03923
GO:0050808	synapse organization	3	2	0.36	0.03923
GO:0051091	positive regulation of sequence-specific DNA binding transcription factor activity	3	2	0.36	0.03923
GO:0051262	protein tetramerization	3	2	0.36	0.03923
GO:0051289	protein homotetramerization	3	2	0.36	0.03923
GO:0090181	regulation of cholesterol metabolic process	3	2	0.36	0.03923
GO:0032504	multicellular organism reproduction	61	12	7.34	0.03954
GO:0002165	instar larval or pupal development	7	3	0.84	0.04016
GO:0003015	heart process	7	3	0.84	0.04016
GO:0007589	body fluid secretion	7	3	0.84	0.04016
GO:0048872	homeostasis of number of cells	7	3	0.84	0.04016
GO:0060047	heart contraction	7	3	0.84	0.04016
GO:0006351	transcription, DNA-dependent	41	9	4.94	0.04017
GO:0009308	amine metabolic process	41	9	4.94	0.04017
GO:0006066	alcohol metabolic process	35	8	4.21	0.04262

GO:0006357	regulation of transcription from RNA polymerase II promoter	12	4	1.44	0.04362
GO:0009968	negative regulation of signal transduction	12	4	1.44	0.04362
GO:0010648	negative regulation of cell communication	12	4	1.44	0.04362
GO:0023057	negative regulation of signaling	12	4	1.44	0.04362
GO:0007165	signal transduction	69	13	8.31	0.04443
GO:0007276	gamete generation	42	9	5.06	0.04652
GO:0009888	tissue development	42	9	5.06	0.04652
GO:0044237	cellular metabolic process	255	35	30.69	0.04950
GO:0031967	organelle envelope	47	12	5.52	0.0033
GO:0031975	envelope	48	12	5.64	0.0040
GO:0005740	mitochondrial envelope	29	8	3.41	0.0116
GO:0005643	nuclear pore	2	2	0.23	0.0135
GO:0046930	pore complex	2	2	0.23	0.0135
GO:0005739	mitochondrion	93	17	10.92	0.0184
GO:0031966	mitochondrial membrane	28	7	3.29	0.0322
GO:0005902	microvillus	3	2	0.35	0.0374
GO:0044429	mitochondrial part	36	8	4.23	0.0432

	rel.het	int.rel	ho.loci	std.het
T2	0.45	-0.73	0.59	1.00
T1	0.93	-0.95	0.34	1.62
M	0.37	-0.73	0.66	0.84
E1	0.38	-0.83	0.60	0.91
E2	0.18	-0.35	0.82	0.50

Additional Files

Additional text

The additional text describes the assembly process and evaluation of assembly quality in further detail. This text also contains figures and tables.

Additional tables

Additional table 1 a lists all data computed on the contig level, including sequences (raw, coding, imputed, protein) additional table 1 b lists only the metadata not including sequences.
Additional table 2 lists high quality SNPs.

Addtional tables 3 list congtigs differentially expressed between male and female worms (a) and European and Asian worms (b).

Additional figures

Additional figure 1: Subgraphs of the GO-ontology categories induced by the top 10 terms identified as enriched in differetn sets of genes. Boxes indicate the 10 most significant terms. Box color represents the relative significance, ranging from dark red (most significant) to light yellow (least significant). In each node the categoy-identifier, a (eventually truncated) description of the term, the significance for enrichment and the number of DE / total number of annotated gene is given. Black arrows indicate a is "is-a" relationship. GO-ontolgy category and the set of genes analysed for the enrchment are indicated in each figure.