Divergence of an introduced population of the Swimbladder-nematode *Anguillicola crassus* - a transcriptomic perspective

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Abstract

The difference of the immune attack on *A. crassus* in the two diffent hosts provides an opportunity to investigate the parasite's response to different "immune environments" on a transcriptomic basis.

To my grandmother Ruth my brother Roman and my wife Silvia

Acknowledgements

I would like to acknowledge the thousands of individuals who have coded for free software and open source projects. It is due to their efforts that code is shared, tested, challenged and improved. Sharing their intellecual property as a general good, they serve progress in science and technology.

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Glossary

DNA Desoxy Ribonucleic Acid; a chemical molecule bearing the heritable genetic information in all life on earth

dpi Days post infection; In infection experiments, a point in time given in days after an individual has been infected

ORF Open Reading Frame; a region in a DNA-sequence begining with a start-codon and not containing a stop-condon. For example a region within a processed mRNA transcript being transcribed into a protein

SNP Single Nucleotide Polymorphism; variation occurring in a single nucleotide between two closely related homlogous sequences. Leading to for example to allelic differences within a population or even the homologous chromosomes in an individual

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Introduction

1.1 The study organism: *Anguillicola crassus*

1.1.1 Ecological significance

Anguillicola crassus Kuwahara, Niimi and Ithakagi 1974 (1, 2) is a swimbladder nematode naturally parasitizing the Japanese eel (*Anguilla japonica*) indigenous to East-Asia. In the last 30 years anthropogenic expansions of its geographic- and host-range to new continents and host-species (all freshwater eels of the genus *Anguilla*) attracted interest of limnologists and ecologists.

First *A. crassus* colonized Europe in the eraly 1980ies and colonized almost all populations of the European eel (*Anguilla anguilla*) in the following decades (reviewed in (3)):

Wielgoss et al. (4) studied the population structure of *A. crassus* using microsattelite markers and inferred details about the colonization process. From the fact that genetic diversity is highest in northern regions of Germany, and gradually declines to the south they concluded a single introduction event (4) to Germany. This is in agreement with the first record of *A. crssus* in 1982 in North-West Germany (5) and the import of Japanese Eels from Taiwan in 1980 having been identified as most likely introduction event (6). Taiwan as the most likely geographical source of the introduction was

At the present day *A. crassus* is found in all but the northernmost population of the European eel in Iceland (7)

1. INTRODUCTION

A second colonization of *A. crassus* succeeded in North-America Since the 1990s populations of the American eel (*Anguilla rostrata*) have been colonized as novel hosts (8, 9, 10) and finally it has been detected in three indigenous *Anguilla* species on the island of Reunion near Madagascar (11).

In Asia, as well as in the introduced ranges, copepods and ostracods serve as intermediate hosts of *A. crassus* (12), in which L2 larvae develop to L3 larvae, infective to the final host. Once ingested by an eel they migrate through the intestinal wall and via the body cavity into the swimbladder wall (13), i.a. using a trypsin-like proteinase(14). In the swimbladder wall L3 larvae hatch to L4 larvae. After a final moult from L4 to preadult the parasites inhabit the lumen of the swimbladder, where they eventually mate. Eggs containing L2 larvae are released via the ductus pneumaticus into the eels gut and finally into the water(15).

Within the novel range and hosts, conspicuously elevated prevalences and intensities of infection occur (reviewed in (3) and (16)). These differences in abundance of *A. crassus* in East Asia compared to Europe are commonly attributed to the different host-parasite relations in the final eel host permitting a differential survival of the larval and the adult parasites (17). Recently, data from experimental infections of European eels with *A. crassus* have been published (18). They show that the parasite undergoes (under experimental conditions) a density-dependent regulation keeping the number of worms within a certain range.

The impact of *A. crassus* on the European eel has been a major focus of research during the past decades. High prevalences of the parasite of above 70% (e.g. (19)), as well as high intesities of infections were reported, throughout the newly colonized area (20). Based on a broad base of work on its epidemiology *A. crassus* can be regarded as a model for parasite introduction and spread (16).

As in the natural host in Asia prevalences and intesities are lower (21), high epidemiological parameters were attributed to the inadequate immune-response of the European Eel (22). Interestingly the differences in the two host also affect the size and life-history of the worm: In European eels the nematodes are bigger and develop and reproduce faster (17). While the Japanese eel is capable of killing larvae of the parasite after vaccination (23) or under high infection pressure (24), only pathological effects such a thikening of the swimmbladder wall (25) have been found in the European eel.

1.1.2 Evolutionary significance

1.1.2.1 Divergence of *A. crassus* populations

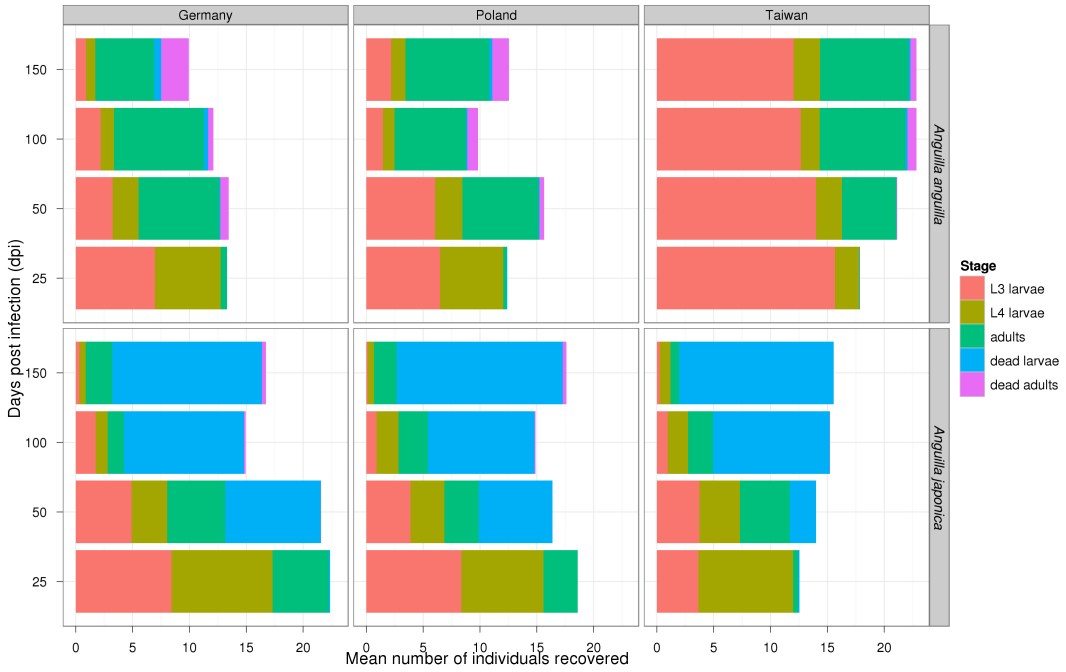

Figure 1.1: Differences in developmental speed - data courtesy of Urszula Weclawski

Today, both theoretical arguments as well as field and laboratory data suggest that evolution, including speciation, can occur very rapidly given the right selective pressure. Such situations provide us with the opportunity of examining how evolution and speciation work at the molecular genetic level (Via 2002).

1.1.2.2 Interest in *A. crassus* based on its phylogeny

In a recent study on the phylogeny of the genus *Anguillicola* we identified *A. crassus* as the most basal species in the genus.

The phylogeny is inconsistent with a

The genus *Anguillicola* holds a phylogenetic position basal to the Spirurina (clade III *sensu* Blaxter (26)), one of 5 major clades of nematodes (27, 28). The Spirurina exclusively exhibit a parasitic lifestyle and comprise improtant human pathogens as well as prominent parasites of livestock (e.g. the Filaroidea and Ascarididae). This

phylogenetic position makes the Anguillicoloidae an interesting system in the endeavour to understand the emergence of parasitism in Spirurina and as an "outgroup" for functional studies of parasitism in this clade. Some functionally interesting genes in this respect are thought to be under diversifying selection in an arms-race between host and parasite(29).

1.1.3 Functional insights from other nematodes used to formulate hypotheses for *A.crassus*

The analysis of ESTs, especially in nematode parasites, has been employed to identify pathogenic factors as potential vaccine candidates in numerous studies. (Blaxter 1995; Blaxter et al. 1996; Daub et al. 2000; Blaxter 2000; Harcus et al. 2004; Mitreva et al. 2004a; Mitreva et al. 2004b; Mitreva et al. 2005).

The complete genome sequence of the nematode Caenorhabditis elegans (The C. elegans sequencing consortium 1998) and Caenorhabditis briggsae (Stein et al. 2003), as well as the draft genomic assembly of Brugia malayi (Ghedin et al. 2007) provide useful sources for mining databases for homologous sequences. Brugia

1.2 Advances in sequencing technology enabeling this study

Recent advances in sequencing technology (often termed Next Generation Sequencing; NGS), provide the opprotunity for rapid and cost-effective generation of genome-scale data.

1.2.1 Pyro-sequencing

The longer read length of 454-sequencing (30) compared to other NGS technologies, allows *de novo* assembly of Expressed Sequence Tags (ESTs) in organisms lacking previouse genomic or transcriptomic data (for a comprehensive list of studies using this approach before Oct 2010 see (31)).

Such transcriptomic datasetes are still less expensive than genomic data-sets in terms sequencing costs and analytical needs.

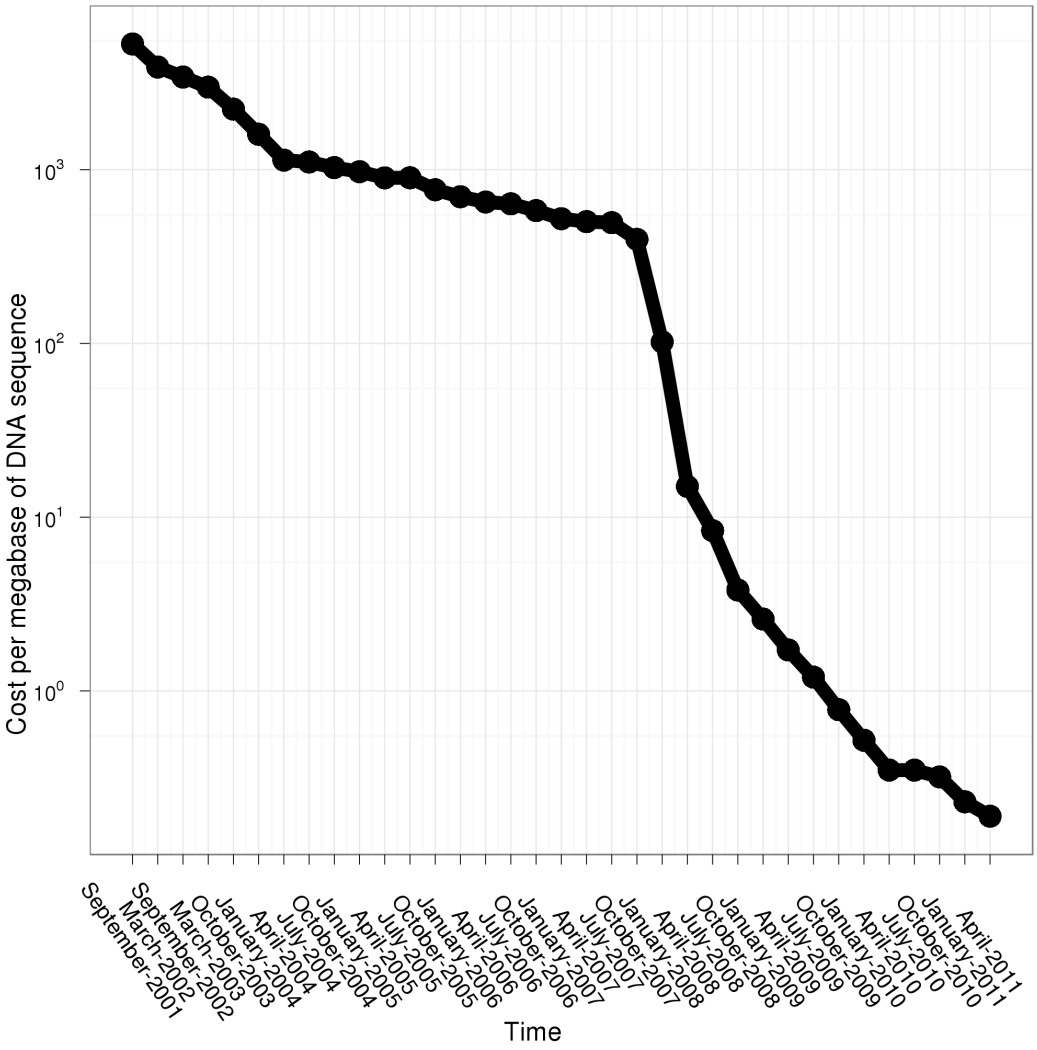

Figure 1.2: Falling seqeuncing costs - Sequencing costs falling due to advances in Solexa-sequencing, due to improved read-length and data-volume on this plattform, Data provided by National Human Genome Research Institute, NHGRI.

1.2.2 Illumina-Solexa sequencing

As shorter read-length but higher throughput of the Illumina-Solexa platform provides superior means for gene expression analyis (32):

Expression-tags (SuperSAGE (33)) provide the benefit of classical SAGE-analysis .

RNA-seq (34)

Gene	GeneID	Length
human latexin	1234	14.9 kbps
mouse latexin	2345	10.1 kbps
rat latexin	3456	9.6 kbps

Table 1.1: title of table - Overview of latexin genes.

2

Aims of the project

2.1 Final aim

The source of my interest in *A. crassus* and the ultimate goal of the study presented here is the identification of .

And followed my interest in

2.2 Preliminary aims

Establishment of transcriptomic and genomic resources,

2. AIMS OF THE PROJECT

3

Pilot sequencing (Sanger method)

3. PILOT SEQUENCING (SANGER METHOD)

4

Pyrosequencing of the *A. crassus* transcriptome

454

5

NlaIII-tag sequencing (Super-SAGE)

5.1 Comparison with pyrosequencing-data

6

Transcriptomic divergence inferred from expression differences in common garden experiments

6. TRANSCRIPTOMIC DIVERGENCE INFERRED FROM EXPRESSION DIFFERENCES IN COMMON GARDEN EXPERIMENTS

7

Discussion

7.0.1 Sanger-method pilot-sequencing

8

Materials & methods

8.1 Sampling of worms from wild eels in Taiwan

During sampling in Taiwan (sampling locations published in (24)) and Germany, single adult *A. crassus* were preserved in RNAlater(Quiagen, Hilden, Germany), after their sex had been determined.

8.2 General RNA-extraction and cDNA synthesis

Total RNA was extracted from single, whole worms using the RNeasy kit (Quiagen, Hilden, Germany), following the manufacturers protocol. Alternatively parts of the liver of the host species *Anguilla japonica*, which also had been preserved in RNAlater were used for RNA extraction, following the same protocol.

The Evrogen MINT cDNA synthesis kit (Evrogen, Moscow, Russia) was then used to amplify mRNA transcripts according to the manufacturers protocol. It uses an adapter sequence at 3' the end of a a poly dT-primer for first strand synthesis and adds a second adapter complementary to the bases at the 5' end of the transcripts by terminal transferase activity and template switching. Using these adapters it is possible to specifically amplify mRNA enriched for full-length transcripts.

8.3 Cloning and Sanger-sequencing

The obtained cDNA preparations were undirectionally cloned into TOPO2PCR-vectors (Invitrogen, Carlsbad, USA) and TOP10 chemically competent cells (Invitrogen, Carls-

bad, USA) were transformed with this construct. The cells were plated on LB-medium-agarose containing Kanamycin (5mg/ml), xGal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl-β-D-1-thiogalactopyranosid). After 24h of incubation at 36 °C cells were picked into 96-well micro-liter-plates containing liquid LB-medium and Kanamycin (5mg/ml) and incubated for another 24h. Subsequently 2ml of the cells were used as template for amplification of the insert by PCR using the primers

Forward M13F(GTAAAACGACGGCCAGT) and

Reverse M13R(GGCAGGAAACAGCTATGACC)

in a concentration of 10μM. The protocol for PCR cycling is shown

Inital denaturation	94 °C	5min	
Denaturation	94 °C	30s	
Annealing	54 °C	45s	35 cycles
Elongation	72 °C	2min	
Filnal Elongation	72 °C	10min	

Table 8.1: PCR protocol for insert amplification

Amplification products were controlled on gel and cleaned using SAP (Shrimp Alkaline Phosphatase) and ExoI (Exonuclease I). Sequencing reactions were performed using the BigDye-Terminator kit and PCR-primers (forward or reverse) in a concentration of 3.5μM and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA). For *A. crassus* the following libraries were prepared:

The original sequencing-chromatographs ("trace-files") were renamed according to the NERC environmental genomics scheme. "Ac" was used as project-identifier for *Anguillicoloides crassus*, "Aj" for *Anguilla japonica*. In *Anguillicoloides* sequences information on the sequencing primer (forward or reverse PCR primer *Anguilla japonica* sequences were all sequenced using the forward PCR primer) was temporarily stored in the middle "library"-field, resulting in names of the following form:

The last field indicates the plate number (two digits), the row (one letter) and the column (two digits) of the corresponding clone. For first quality trimming trace2seq, a tool derived from trace2dbEST (both part of PartiGene (35)) was used, briefly it

performs quality trimming using phred(36) and trimming of vector sequences using cross-match(37). The adapters used by the MINT kit were trimmed by supplying them in the vector-file used for trimming along with the TOPO2PCR-vector. After processing with trace2seq additional quality trimming was performed on the produced sequence-files using a custom script. This trimming was intended to remove artificial sequences produced when the sequencing reaction starts at the 3' end of the transcript at the poly-A tail. These sequences typically consist of numerous homo-polymer-runs throughout their length caused by "slippage" of the reaction. The basic perl regular expression used for this was:

```
/(.*A{5,}|T{5,}|G{5,}|C{5,}.*){$lengthfac,}/g
```

Where `$lengthfac` was set to the length of the sequence devided by 70 and rounded to the next integer. So only one homo-polymer-run of more then 5 bases was allowed per 105 bases. Results of this screening were checked by blasting the sequences excluded as artificial against nempep4, a nematode rRNA database and a fish-protein database. Two sequences which were identified as false positives (hitting proteins in nempep4) were moved manually to the sequences still categorized as good. These were screened against *Anguillicoloides* rRNA or fish rRNA using cross-match(37) with standard parameters for screening. Finally GS content was tabulated for the sequences intended for submission and screening statistics were calculated.

After this step sequences were screened for host contamination by a comparison of BLAST searches against nempep4 and a fish protein database. Sequences producing better bit scores againt fish proteins than nematode proteins were removed.

Only the trace-files corresponding to the sequences still regarded as good after this step were processed with trace2dbEST. Additionally to the processing of traces already included in trace2seq sequences were preliminary annotated using BLAST versus the NCBI-NR non-redundant protein database and a EST-submission-file was produced. This file parsed for the information on the sequencing primer (stored in the library-field) and the corresponding primer-entries in the file were replaced.

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Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from XXX to YYY under the supervision of PI at ZZZ.

CITY,