Comprehensive analysis of human hookworm secreted proteins using a proteogenomic approach

Logan J[a], Manda SS[b,c], Choi YJ[d], Field M[a], Eichenberger RM[a], Mulvenna J[e], Nagaraj SH[f], Fujiwara RT[g], Gazzinelli-Guimaraes P[g], Bueno L[g], Mati V[h], Mitreva M[d], Sotillo J[a#], Loukas A[a#]

[a]Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia

[b]Cancer Data Science Group, ProCan, Children's Medical Research Institute, Faculty of Medicine and Health, University of Sydney, Westmead, NSW, Australia

[c]LifeBytes India Pvt Ltd, Whitefield, Bangalore, India

[d]McDonnell Genome Institute, Washington University School of Medicine, St. Louis, Missouri, USA

[e]QIMR-Berghofer Medical Research Institute, Brisbane, QLD, Australia

[f]Institute of Health and Biomedical Innovation and Translational Research Institute, Queensland University of Technology, Brisbane, QLD, Australia

[g]Department of Parasitology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Brazil

[h]Department of Health Sciences, Universidade Federal de Lavras, Lavras, Brazil.

[#] Both authors contributed equally to this work

Corresponding Authors:

Prof. Alex Loukas. Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Cairns, 4878, Queensland, Australia. Email: alex.loukas@jcu.edu.au

Dr. Javier Sotillo. Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Cairns, 4878, Queensland, Australia. Email: javier.sotillo@jcu.edu.au

Running title: Proteomic analysis of *Necator americanus* secreted proteins

Abbreviations:

ASP – Aspartyl

ASPR – Ancylostoma secreted protein related

CAP – Cysteine-rich secretory protein

CDS – Coding sequence

cRAP – Common repository of adventitious proteins

CRISP - Cysteine-rich secretory protein

CYS – Cysteine

DP – Declustering potential

DUF – Domain of unknown function

emPAI – Exponential modified protein abundance index

ES – Excretory/Secretory

FDR – False discovery rate

GO – Gene ontology

GSSPs – Genome search specific peptides

IAM – Iodoacetamide

iTOF – Interactive tree of life

MET – Metallo

ML – Maximum-likelihood

NK – Natural killer

NP – Number of peptides

RO – Reverse osmosis

SCP/TAPS – Sperm-coating protein

SER – Serine

SFG – Six-frame translated genome

SP – Signal peptide

SRA – Sequence read archive

TD – Transmembrane domain

Summary

The human hookworm *Necator americanus* infects more than 400 million people worldwide, contributing substantially to the poverty in these regions. Adult stage *N. americanus* live in the small intestine of the human host where they inject excretory/secretory (ES) products into the mucosa. ES products have been characterized at the proteome level for a number of animal hookworm species, but until now, the difficulty in obtaining sufficient live *N. americanus* has been an obstacle in characterizing the secretome of this important human pathogen. Herein we describe the ES proteome of *N. americanus* and utilize this information to conduct the first proteogenomic analysis of a parasitic helminth, significantly improving the available genome and thereby generating a robust description of the parasite secretome. The genome annotation resulted in a a revised prediction of 3,425 fewer genes than initially reported, accompanied by a significant increase in the number of exons and introns, total gene length and the percentage of the genome covered by genes. Almost 200 ES proteins were identified by LC-MS/MS with SCP/TAPS proteins, 'hypothetical' proteins and proteases among the most abundant families. These proteins were compared to commonly used model species of human parasitic infections, including *Ancylostoma caninum*, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. Our findings provide valuable information on important families of proteins with both known and unknown functions that could be instrumental in host-parasite interactions, including protein families that might be key for parasite survival in the onslaught of robust immune responses, as well as vaccine and drug targets.

Introduction

Hookworm infection is one of the most pertinent and life-limiting parasitic infections worldwide, affecting more than 400 million people in tropical regions of Asia, Africa and South America (1, 2). Chronic infection with hookworms results in fatigue, abdominal pain, diarrhea, weight loss and anemia (3). In children, it can cause growth retardation and impairments in cognitive development (4), and in pregnant women these infections lead to poor birth outcomes including low birth weight, increased perinatal morbidity and mortality (5, 6). Moreover, hookworm infections result in 3.2 million disability-adjusted life years lost annually (7). Hookworm infection therefore contributes significantly to widespread poverty in the majority of endemic regions (8).

The life cycle of the most widespread of the anthropophilic hookworms, *Necator americanus*, is direct, with no intermediate hosts involved. Eggs are passed out of the body in the feces and, under favorable conditions, hatch releasing first-stage (L1). Larvae undergo several molts to reach the infective third (L3) stage which can penetrate human skin (9). Upon infection the parasite migrates through the circulatory system to the lungs, where it moves up the trachea and is eventually swallowed, thus commencing its final sojourn through the gastrointestinal tract to reside in the small intestine where mature adult worms can live for up to 10 years (9). The adult stage of *N. americanus* produces macromolecules known as excretory/secretory (ES) products, which consist of a battery of proteins that have evolved to interact with human host tissues and facilitate parasitism (10). These ES products have the potential to not only be targeted as potential vaccine and diagnostic candidates, but also to shed light on how these parasites evade immune destruction (11-14).

Despite their potential biotechnological utility, only a limited number of *N. americanus* ES proteins have been described to date, and most of them have been identified as cDNAs based on their homology to proteins from more readily accessible hookworm species from animals such as *Ancylostoma caninum* (15). In terms of vaccine antigens, a handful of *N. americanus* ES products including glutathione-S-transferases, aspartic proteases and sperm-coating proteins/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS), have been identified at the cDNA level, and vaccine efficacy of recombinant proteins assessed in animal models and phase 1 clinical trials (13, 16, 17). SCP/TAPS, also referred to as venom allergen-like (VAL) or Activation-associated Secreted Proteins (ASPs) (Pfam accession number no. PF00188) have been reported from many helminths, but appear to be significantly expanded in the genomes and secreted proteomes of gut-resident clade IV and V parasitic nematodes, including the hookworms (11, 18-20).

Considerably little is known about the roles of *N. americanus* proteins in immunoregulation compared with many of the parasites that serve as animal models for human infections. Hsieh and colleagues reported an *N. americanus* protein(s) which selectively bound Natural Killer (NK) cells resulting in IL-2- and IL-12-dependent IFN-γ production, although the identity of the protein was not determined (21). Calreticulin, a protein from the ES products of *N. americanus*, was identified as having an immunomodulatory role through inhibition of the hemolytic capacity of C1q, a human complement protein (22). The relative paucity of functional information on *N. americanus* proteins can be attributed, at least in part, to the difficulty in obtaining parasite material and the absence until 2014 of the published *N. americanus* draft genome. Analysis of the 244 Mb draft genome and the predicted 19,151 genes (11), provided important information about the molecular mechanisms and pathways by which *N. americanus* interacts with its human host. In agreement with published transcriptomes of *N. americanus* and genomes/transcriptomes of other hookworm species, a select number of protein families were over-represented, including SCP/TAPS proteins and different mechanistic classes of proteases with various functions including hemoglobinolysis, and tissue penetration (23-28). Of the >19,000 predicted genes reported in the draft *N. americanus* genome, 8,176 genes had no known InterPro domain. Additionally, more than half of the total proteins had either no blast homology to any gene from the NCBI database (10,771 proteins) or shared identity with a 'hypothetical' protein (3,043 proteins). These results highlight the importance of further annotation and refinement of the *N. americanus* genome (29).

In general, the practicality of genomic sequence data is dependent on the accuracy of gene annotation as well as the availability of functional, expression and localization information (30). The high-throughput methods used when annotating a genome are prone to errors, therefore, to validate the predicted protein-coding genes, an analysis of the proteome is essential. Mass spectrometry provides useful data that can be used in a proteogenomic approach to improve genome annotation and identify novel peptides containing predicted protein sequences (31).

Herein we perform the first proteogenomic analysis of a parasitic helminth, while also significantly improving the genome annotation and comprehensively characterizing the ES proteome of adult *N. americanus*. Our findings provide valuable information on important families of proteins with both known and unknown functions that could be instrumental in host-parasite interactions, including protein families that might be key for parasite survival and protection of the host against excessive immunopathology. Characterization of these proteins will be useful for the identification of vaccine and drug targets and diagnostics for hookworm infection.

Experimental procedures

Ethics

Ethical approval for hamster animal experimentation to obtain *N. americanus* adult worms was obtained from the Federal University of Minas Gerais, Brazil (Protocol# 51/2013). Ethical approval for human experimental infection with *N. americanus* and subsequent culturing of L3 was obtained from the James Cook University Human Research Ethics Committee (ID# H5936).

Parasite material

Adult *N. americanus* were manually isolated from the intestines of experimentally infected golden hamsters (*Mesocricetus auratus*) upon euthanasia. For the isolation and purification of ES products, the worms were washed 3 times in phosphate-buffered saline (PBS) before being cultured overnight in a humidified incubator at 37°C, 5% CO_2, in RPMI 1640 (100U/ml penicillin, 100 µg/ml streptomycin sulphate, 0.25 µg/ml amphotericin B). The supernatant was collected the following day and debris was removed by centrifuging the concentrated samples at 1,500 g for 3 minutes in a benchtop microfuge. ES products were concentrated with a 3 kDa cut-off Centricon filter membrane (Merck Millipore) and samples were stored at -80°C until use. For somatic adult extracts, data was used from Tang *et al* (11). In brief, whole worms were ground under liquid nitrogen and solubilized using lysis buffer (1.0% (v/v) Triton X-100 in 40 mM Tris, 0.1% (w/v) SDS, pH 7.4). The extract was filtered through 20µm filter before fractionation.

For isolation of *N. americanus* L3, stool samples were collected from infected human volunteers and cultured as follows. Reverse osmosis (RO) water was added to the stool sample until a thick paste was formed. This paste was then distributed onto moistened Filter Paper (VWR, Standard Grade, 110 mm) in Petri dishes (Sarstedt, 150 mm) and placed in a 25°C incubator for 8 days. Following incubation, the edges of each plate were gently rinsed with RO water to obtain clean L3 preparations. Somatic extracts were prepared by adding 100 µl lysis buffer (3 M Urea, 0.2% SDS, 1% Triton X, 50 mM Tris-HCl) to approximately 6,000 larvae before repeated vortexing and sonication (4°C, probe sonicator, pulse setting) to digest the larvae. The extract was passed through a 0.45 µm filter (Millipore) before in-gel fractionation.

SDS-PAGE fractionation and in-gel trypsin digestion

A total of 30 µg of *N. americanus* adult ES products was buffer exchanged into 50 mM NH_4HCO_3, freeze-dried and resuspended in Laemmli buffer. The sample was boiled at 95°C for 5 minutes and electrophoresed on a 12% SDS-PAGE gel for 40 minutes at 150V. The gel was stained with Coomassie Blue and 20 pieces (approximately 1 mm thick) were cut and placed into Eppendorf tubes. For the in-gel digestion, slices were de-stained and freeze-dried before incubating them at 65°C for 1 h in reduction buffer (20 mM dithiothreitol (DTT), Sigma, 50 mM NH_4HCO_3). Samples were then alkylated in 50 mM iodoacetamide (IAM, Sigma), 50 mM NH_4HCO_3 for 40 minutes at 37°C, washed three times with 25 mM NH_4HCO_3 and digested with 20 µg/ml of trypsin (Sigma) by incubating them for 16 h at 37°C with gentle agitation. Digestion was stopped and peptides were released from the slices by adding 0.1% TFA, 70% acetonitrile. This step was repeated 3 times with pooling of the corresponding supernatants to maximise peptide recovery for each sample. Finally, each sample was desalted with a ZipTip (Merck Millipore) and stored at -80°C until use.

In-solution trypsin digestion and off-gel fractionation

A total of 70 µg of each *N. americanus* extract (L3 somatic and adult ES products) was buffer exchanged into 50 mM NH_4HCO_3 before adding DTT to 20 mM and incubating for 10 minutes at 65°C. Alkylation was carried out by adding IAM to 55 mM and incubating for 45 minutes at room temperature in the dark. Samples were digested with 2 µg of trypsin by incubating for 16 h at 37°C with gentle agitation. Following trypsin digestion, peptides were fractionated using a 3100 OFFGEL Fractionator (Agilent Technologies) according to the manufacturer's protocol with a 24-well format as described previously (19). In brief, Immobiline DryStrip pH 3-10 (24 cm) gel strips were rehydrated in rehydration buffer in the assembled loading frame. Digested peptides were diluted in dilution buffer to a volume of 3.6 ml and loaded equally across the 24 well cassette. The sample was run at a current of 50 μA until 50 kilovolt hours (kVh) had elapsed. Upon completion of the fractionation, samples were collected, desalted with ZipTip and stored at -80°C until use.

Mass Spectrometry

The extracts were analyzed by LC-MS/MS on a Shimadzu Prominence Nano HPLC (Japan) coupled to a Triple Tof 5600+ mass spectrometer (SCIEX, Canada) equipped with a nano electrospray ion source. Fifteen (15) µl of each extract was injected onto a 50 mm x 300 µm C18 trap column (Agilent Technologies, Australia) at 60 µl/min. The samples were de-salted on the trap column for 6 minutes using 0.1% formic acid (aq) at 60 µl/min. The trap column was then placed in-line with the analytical nano HPLC column, a 150 mm x 100 µm 300SBC18, 3.5 µm (Agilent Technologies, Australia) for mass spectrometry analysis. For peptide elution and analysis the nano-HPLC pump was initially held at 2% solvent B for 6 minutes followed by a linear gradient of 2-40% solvent B over 80 minutes at 500 nl/minute flow rate and then a steeper gradient from 40% to 80% solvent B in 10 minutes was applied. Solvent B was held at 80% for 5 minutes for washing the column and returned to 2% solvent B for equilibration prior to the next sample injection. Solvent A consisted of 0.1% formic acid (aq) and solvent B contained 90/10 acetonitrile/ 0.1% formic acid (aq). The ionspray voltage was set to 2200V, declustering potential (DP) 100V, curtain gas flow 25, nebulizer gas 1 (GS1) 12 and interface heater at 150°C. The mass spectrometer acquired 250 ms full scan TOF-MS data followed by 20 by 250 ms full scan product ion data in an Information Dependent Acquisition (IDA) mode. Full scan TOF-MS data was acquired over the mass range 300-1600 and for product ion ms/ms 80-1600. Ions observed in the TOF-MS scan exceeding a threshold of 150 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, MS/MS spectra of the resultant 20 most intense ions. The data was acquired using Analyst TF 1.6.1 (SCIEX, Canada).

Proteogenomics

The mass spectrometry raw data was searched against the *N. americanus* protein database using SequestHT algorithm in Proteome Discoverer 2.1 (Thermo Scientific, Bremen, Germany). Trypsin was used as the protease, allowing a maximum of two missed cleavages. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was included as variable modifications. The minimum peptide length was specified as 6 amino acids. The mass error of parent ions was set to 10 ppm, and for fragment ions it was set to 0.05 Da. Protein inference was based on the rule of parsimony and required one or more unique peptides. False Discovery Rate (FDR) of 1% at peptide and protein levels was applied. The unassigned spectra from the protein database search were further searched against the six-frame translated genome database of *N. americanus* (11). The genome sequences were downloaded from WormBase ParaSite release 9 (http://parasite.wormbase.org/Necator_americanus_prjna72135/Info/Index) in FASTA format and translated in all six frames using in-house python scripts. All the sequences greater than 10 amino acids between any two stop codons were added to the database. The search was again performed using SequestHT with precursor mass tolerance of 50 ppm and fragment ion tolerance of 1 Da. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was included as variable modifications. Results were obtained at 1% FDR for both protein and peptide level.

The identified peptides in the search against the six-frame translated genome were mapped back to the protein database using standalone BLAST. Any sequences that mapped 100% to the protein database were discarded. The filtered peptides were mapped to the *N. americanus* genome using the standalone tblastn program (11). Peptide identifications that unambiguously mapped to a single region in the genome, also known as Genome Search Specific Peptides (GSSPs), were considered to perform proteogenomics-based annotation of novel coding regions. The known gene annotation GFF3 was downloaded from WormBase ParaSite for *N. americanus* (11). Using in-house scripts, we categorized the GSSPs into various categories: intergenic, intronic, exon-extension, alternative frame, N-terminal extensions or repeat regions with respect to the known regions from GFF3. Additionally, for all the intergenic peptides, we determined if there was a potential open reading frame (ORF) within the stretch of amino acids. Each of the spectra was further manually validated.

Genome annotation

The genome annotation from *N. americanus* (11) was updated using the MAKER pipeline v2.31.8 (32). The genome assembly (GenBank assembly accession: GCA_000507365.1) was softmasked for repetitive elements with RepeatMasker v4.0.6 using a species-specific repeat library created by RepeatModeler v1.0.8, RepBase repeat libraries (33) and a list of known transposable elements provided by MAKER (32). From the NCBI Sequence Read Archive (SRA), *N. americanus* RNA-Seq data (11) (Adult: SRR609895, SRR831085, SRR892200; L3: SRR609894, SRR831091, SRR89220) were obtained. After adapter and quality trimming using Trimmomatic v0.36 (34), RNA-Seq reads were aligned to the genome using HISAT2 v2.0.5 (35) with the --dta option and subsequently assembled using StringTie v1.2.4 (36). The resulting alignment information and transcript assembly were used by BRAKER (37) and MAKER pipelines, respectively, as extrinsic evidence data. In addition, protein sequences from SwissProt UniRef100 (38) and WormBase ParaSite WS258 (39) (*Ancylostoma ceylanicum* PRJNA231479, *Brugia malayi* PRJNA10729, *Caenorhabditis elegans* PRJNA13758, *Onchocerca volvulus* PRJEB513, *Pristionchus pacificus* PRJNA12644, *Trichinella spiralis* PRJNA12603 and *Strongyloides ratti* PRJEB125) were provided to MAKER as protein homology evidence. Following the developer's recommendation (40), the protein-coding gene models of Tang *et al.* (11) were passed to MAKER as pred_gff to update the models by adding new 3' and 5' exons, additional UTRs, and merging split models. This method, however, cannot change internal exons nor create new annotations where evidence suggests a gene but no corresponding model is previously present. To address this shortcoming, additional *ab initio* gene predictions were generated using BRAKER v2.0.1 (37) and passed to MAKER so that the intron-exon model that best matched the evidence could be included in the final annotation set. Within the BRAKER pipeline, the gene prediction tools GeneMark (41) and AUGUSTUS (42) were trained utilizing the *N. americanus* RNA-Seq alignment and protein homology information from *C. elegans*. The GFFPs identified in the present study were used to confirm the validity of proposed annotation changes and resolve competing gene predictions through manual curation. Gene models with no evidence support were not included in the final annotation build to reduce false positives in the existing annotations. However, *ab initio* gene predictions that encoded Pfam domains as detected by InterProScan v5.19 (43) were rescued to enhance overall accuracy by balancing sensitivity and specificity (32, 44). The completeness of the annotated gene set was assessed using BUSCO v3.0 with Eukaryota-specific single copy orthologs (OrthoDB v9) (45).

Protein Identification

Mascot version 2.5 (Matrix Science), X!Tandem (The Global Proteome Machine Organisation) version Jackhammer and Comet v2014.02 rev.2 were used to analyze data from the mass spectrometer. Searches were carried out against a database comprised of either the updated genome annotation provided with this study, or the *N. americanus* genome (11), both appended to the common repository of adventitious proteins (cRAP; http://www.thegpm.org/crap/) database (to detect potential contamination). The following parameters were used: enzyme, trypsin; variable modifications, oxidation of methionine, carbamidomethylation of cysteine, deamidation of asparagine and glutamine; maximum missed cleavages, 2; precursor ion mass tolerance, 50 ppm; fragment ion tolerance 0.1 Da; charge states, 2+, 3+ 4+. A FDR of 0.1% was applied, and a filter of greater than 2 significant unique sequences was used to further improve the robustness of data. The mass spectrometry data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010669.

Bioinformatic Analysis of Proteomic Sequence Data

Gene ontology (GO) annotations were assigned using the program Blast2GO and Pfam analysis was performed using HMMER (46). Pfam domains were detected at the $P<0.001$ threshold for the HMMER software. Putative signal peptides were predicted with SignalP v4.1 and transmembrane domains with TMHMM v2.0 (47, 48). REViGO, an online tool, was used to summarise and plot GO terms (49). UpSetR was used to group proteins based on whether they had a GO term with one, two or all of the GO categories (biological process, molecular function or cellular process) (50).

Similarity analysis

A similarity analysis was carried out based on the Parkinson and Blaxter method using an in-house script (26, 51). Given the difficulty of working with *N. americanus* specifically (in terms of accessibility to samples and establishment of life cycle in other hosts), other hookworms are frequently used to model this human parasite. Data from the secreted proteome of adult *N. americanus* (described herein) was compared to the published secreted proteomes from related adult nematode species including *H. polygyrus*, *A. caninum* and *Nippostrongylus brasiliensis* (15, 19, 52).

Protein family similarity visualization

H. polygyrus, *A. caninum* and *N. brasiliensis* SCP/TAPS and protease protein sequences were obtained from their respective published secreted proteomes (15, 19, 52). These sequences were aligned with adult *N. americanus* homologous protein sequences using BLAST. Significant sequence alignments were visualized using Circos (53).

Phylogenetic Analyses

SCP/TAPS proteins were identified from published secretomes of 6 species of parasitic helminths including *A. caninum, Ascaris suum, H. polygyrus, N. brasiliensis, Trichuris muris* and *Toxocara canis.* SCP/TAPS proteins were identified from *N. americanus* ES products using the proteome data generated in this study. Sequences were sorted into single and double SCP/TAPS domain proteins for individual phylogenetic analysis due to distinct differences described previously (54). A list of the proteins and their respective sequences used for this analysis can be found in

Supplemental Data 1. A multiple sequence alignment was carried out using the alignment program MUSCLE. Outliers with poor alignment (long unaligned regions) were detected and filtered out using ODseek. PhyML, a phylogeny software, was used for a maximum-likelihood (ML) phylogenetic analyses of SCP/TAPS amino acid sequences. The tree was visualized with The Interactive Tree of Life (iTOF) online phylogeny tool (https://itol.embl.de/) (55).

Results

Proteogenomic analysis and genome annotation

Different proteomes from two life stages (infective L3 somatic extract and adult somatic extracts and ES products) of *N. americanus* were analysed by mass spectrometry in a 5600 ABSciex Triple Tof to perform a proteogenomic analysis of the hookworm genome. After excluding peptides that mapped accurately to the existing protein database entry, a total of 218 novel peptides were identified that did not match any protein sequences of *N. americanus* contained in the annotations of Tang *et al* (11). Of these 218 novel peptides, 83 were found exclusively in adult somatic extracts, 50 exclusively in L3, and 67 exclusively in adult ES, while 10 peptides were found in both the adult somatic extract and ES products, and 8 were found in both adult and larval somatic extracts. No common peptides were identified from adult ES products and larval somatic extract.

Newly identified peptides could be grouped into the following six categories: (1) peptides mapping to intergenic regions; (2) peptides mapping to introns; (3) peptides mapping to alternative reading frames; (4) peptides extending gene boundaries (exon extensions); (5) peptides mapping to N-terminal extensions; and (6) peptides mapping to repeat regions (not gene) regions (Figure 1). Of the identified peptides, more than half belonged to group 1, 18% to group 2, 15% to group 3 and a small number to groups 4, 5 and 6 (Figure 1). Group 1 peptides were further analyzed for an

ORF including whether or not they contained a methionine; see Supplemental data 2. From this data, one-third of group 1 peptides were found to overlap with a viable ORF, with the shortest having just 21 amino acids and the longest 381 amino acids. The translated protein sequences with their respective peptides, six-frame translated genome (SFG) ID and ORF length are provided in Supplemental data 3.

These results highlighted the need for improving the previously published gene models, so we updated the genome annotation of *N. americanus* using a new RNA-Seq based gene calling pipeline as outlined in the Materials and Methods (Table 1). The total number of predicted *N. americanus* genes decreased by 3,425, with substantial increases in both the number of exons and introns. The total coding sequence (CDS) length increased by 2.22 Mb with the mean gene length (including introns and UTRs) nearly doubling from 4.3 kb to 8.1 kb. Furthermore, the percentage of the genome covered by genes increased by 18.3%, and the percentage of detected BUSCOs among the predicted genes increased from 95.7% to 97.4% (with 31% reduction in the number of fragmented BUSCOs). While these improvements are attributable in most part to the use of more sophisticated genome annotation methods utilizing RNA-Seq data and the inclusion of more extensive, up-to-date homologous protein databases, the peptide sequences generated in this study contributed directly to the refinement of 14 gene models. The newly annotated, improved gene models were used in subsequent proteomic analysis of ES products, and are publicly available on Nematode.net (56, 57).

Analysis of the ES products from N. americanus adult worms

A comprehensive analysis of the ES products from adult worms was carried out using in-gel and off-gel fractionation and the tryptic peptides were analyzed using LC-MS/MS. Mascot, X!Tandem and Comet searches were carried out against a database including the predicted proteins from the annotated *N. americanus* genome available in this study and the cRAP sequences available at http://www.thegpm.org/crap/. A total of 186 and 141 proteins were identified using Mascot and X!Tandem/Comet, respectively. All ES proteins were identified with at least two unique peptides, at a 99.0% probability and FRD of 0.1%. The two search methods combined found a total of 198 proteins, Supplemental data 4). These 198 proteins were obtained using the updated genome annotation generated as part of this study. In comparison, using the first version of the annotated genome sequence we identified 203 proteins using the same analytical software (11). Using the exponential modified protein abundance index (emPAI) and the newly annotated genome, the most abundant proteins in the ES products of *N. americanus* adult worms were ranked, and the top 30 are shown in Table 2. One emPAI value was listed for each of the digestion methods used (Ingel and Offgel) and their combined value was used to rank the proteins.

The conserved Pfam domains of the 198 ES proteins identified were analyzed. The most abundant protein family in the ES products was the SCP/TAPS family with 54/198 proteins containing a single or double cysteine-rich secretory protein family (CAP) domain (PF00188) (Figure 2B). The top 10 most abundant protein families are displayed in Figure 2B. Many of these proteins were described by Blast2GO as 'SCP-partial' or 'SCP-like', but for a more standardized annotation in our analysis we have grouped them all as 'SCP/TAPS'. The second most frequently represented group (with 42 proteins) were proteins with one or more domains of unknown function (DUF). Other abundant families included Ancylostoma secreted protein related (ASPR) proteins and metalloproteases with 35 and 9 of each identified respectively (Supplemental data 5). Despite some published reports classifying ASPRs as SCP/TAPS proteins, they are a diverse set of secreted cysteine rich proteins based on Pfam annotation and therefore we have grouped them separately

(28). Of the 198 identified ES proteins, 51% contained a predicted signal peptide. Supporting the accuracy of the new gene model, 96 of the identified adult ES proteins were predicted to contain a signal peptide compared to just 75 using the previous genome annotation.

The adult *N. americanus* ES proteins were annotated using Blast2GO (46). In total, 30 GO terms were identified (following the removal of parent child redundancy) belonging to one of the three GO database categories: biological processes, molecular function or cellular component (Figure 2A, for raw data see Supplemental data 6). Blast2GO returned biological process GO terms for 87/198 proteins, molecular function GO terms for 99/198 proteins and cellular component GO terms for 83/198 proteins (Figure 2A). The most prominent biological process term was proteolysis (Figure 2D), with 15% (29 proteins) of total ES products being involved in a proteolytic process. The most prominent single molecular function term was "hydrolase activity" followed by "peptidase activity" and "metal ion binding" (Figure 2C). Interestingly, 50/198 proteins did not return any GO terms (Figure 2A). Of these, SCP/TAPS proteins made up 64% with no known biological process, molecular function, or cellular component. This highlights a significant knowledge gap surrounding SCP/TAPS produced by helminths.

Similarity analysis of the ES products from different gastrointestinal nematode species

A similarity analysis of ES proteomic data from *N. americanus* and three of the most commonly used animal models for human hookworms, *A. caninum*, *H. polygyrus* and *N. brasiliensis* was carried out (Figure 3). A total of 15, 10 and 1 *N. americanus* ES proteins had unique homology to ES proteins from *A. caninum*, *H. polygyrus* and *N. brasiliensis* respectively. This included one SCP/TAPS protein (NAME_13724) which was similar only to an *H. polygyrus* protein, while 3 of the proteins were similar only to *A. caninum* ES proteins with domains of unknown function (NAME_07734, NAME_09181, NAME_09182). Seven proteins shared homology with only *A. caninum* and *H. polygyrus* proteins, 11 shared homology with only *A. caninum* and *N. brasiliensis* proteins and 7 shared homology with only *H. polygyrus* and *N. brasiliensis* proteins. One hundred and twenty-two *N. americanus* proteins shared different degrees of homology with proteins from *A. caninum*, *H. polygyrus* and *N. brasiliensis*.

From the 54 SCP/TAPS proteins found in *N. americanus* ES products, one (NAME_13724) shared homology with a protein found in only *H. polygyrus*, while another SCP/TAPS protein (NAME_15177) shared homology with proteins from both *A. caninum* and *N. brasiliensis*. Fifty-one (51) of 54 SCP/TAPS proteins were similar to all compared species, leaving a single SCP/TAPS protein (NAME_11218) which did not have homology to any SCP/TAPS protein from the compared species. Twenty-five *N. americanus* ES proteins did not have homology to any proteins in the secretome of *A. caninum*, *H. polygyrus* or *N. brasiliensis.* Notable proteins among these 25 were NAME_01848 (aspartyl protease) and NAME_05081 (zinc metalloprotease).

Of the 26 proteases in the *N. americanus* ES products, 22 had homologs in all compared species, while one serine protease (NAME_06735) only had homologs in *H. polygyrus* and *A. caninum* and one metalloprotease (NAME_00535, peptidase family M1) only had homologs in the ES products of *A. caninum*, and *N. brasiliensis*. One zinc metalloprotease (NAME_05081) and one serine protease (NAME_01250) were only found in the ES products of *N. americanus* and therefore did not have any similarity to ES products from the other species.

Homology analysis of SCP/TAPS and proteases in the ES products of N. americanus

Since SCP/TAPS proteins and proteases from *N. americanus* numerically dominate the ES protein dataset and likely play key roles in infection, migration and parasite establishment, we performed an in-depth analysis of these families of proteins between the human and three model gastrointestinal nematode species. Adult *N. americanus* ES SCP/TAPS protein sequences were aligned with homologs from *H. polygyrus*, *A. caninum* and *N. brasiliensis* ES SCP/TAPS protein sequences using BLAST. Sequences which aligned with maximum scores >36 were visualized using Circos (Figure 4A). SCP/TAPS from *N. americanus* are more similar to *A. caninum* than *H. polygyrus* or *N. brasiliensis*, as denoted by thicker, darker ribbons (Figure 4A). The sequences, their homologs and the corresponding blast scores are detailed in Supplemental data 7. As with the similarity analysis, NAME_11218 had no significant alignment to any of the compared species.

A similar analysis was performed for the proteases from each of the aforementioned species (Figure 4B). These proteases have been grouped together into their respective mechanistic classes: aspartyl (ASP), cysteine (CYS), metallo (MET) or serine (SER) proteases. In general, all three comparator species had high protease sequence homology to metalloproteases from *N. americanus*. Aspartyl protease sequences were more similar between *H. polygyrus*, *N. brasiliensis* and *N. americanus* than sequences from *A. caninum*. It is interesting to note that *N. americanus* contained more aspartyl proteases than the other nematode species analyzed (*N. americanus* – 8; *A. caninum* – 4; *H. polygyrus* – 6; *N. brasiliensis* – 6). Serine proteases had the lowest sequence homology across all of the compared species and protease subclasses.

Phylogenetic analysis of SCP/TAPS proteins

SCP/TAPS sequences of 6 parasitic nematodes were obtained from published secretomes and compared to *N. americanus* SCP/TAPS identified in this study. Sequences were grouped by whether they had a single or double domain and then aligned using MUSCLE and PhyML. From the 7 total species, 232 SCP/TAPS were reported with 134 single-domain proteins and 98 double-domain. For the single SCP/TAPS-domain proteins an unrooted tree was generated. The analysis identified seven main clades. Three of these clades consisted entirely of sequences from *N. americanus* and *A. caninum*, while sub-clades in 3 of the other main clades followed a similar trend. A majority of *T. muris* single-domain sequences formed a sub-clade with *A. suum* and *T. canis*, grouping together the non-clade V helminths. An unrooted tree was also generated for double domain sequences. The analysis identified five main clades. *N. americanus* SCP/TAPS clustered almost exclusively with sequences from *A. caninum* again, representing one main clade and several sub-clades. *H. polygyrus* and *N. brasiliensis* also formed a number of distinct sub-clades. *T. canis*, the only non-clade V helminth, was reported to only produce one double-domain SCP/TAPS protein in its ES products which was not closely related to any of the compared sequences. Interestingly, no double-domain SCP/TAPS were reported for *A. suum* or *T. muris*. Another trend across both single and double domain trees was the diversity of evolution between SCP/TAPS within a single species. For example, the single-domain tree included a sub-clade of 7 *N. americanus*-only sequences while other *N. americanus* sequences had more similarity to mouse hookworm sequences.

Discussion

N. americanus affects more than 400 million people worldwide and is the most important soil transmitted helminth in terms of morbidity (2). The genome of *N. americanus* was sequenced in 2014, providing an important dataset to facilitate efforts to combat hookworm disease; however, the tools available at that time for annotating genes from parasitic helminths were limited. For instance, the number of proteins with a top hit to a ‘hypothetical protein’ present in the original genome annotation was 3,043, corresponding to 15.8% of the total predicted proteins (11). In addition, inferring gene and protein functions for parasitic nematodes is a major challenge as most species are genetically intractable and databases and algorithms are biased towards (free-living) model nematodes (29). Proteogenomics is a relatively new approach in which proteomic data is used to improve genome annotation (31, 58), and although it had never been applied to parasitic helminths (until now), its potential utility in this area has been suggested (59). High-throughput sequencing and gene prediction tools are prone to false-negative and false-positive predictions which can lead to missed genes, false exons or exon boundaries and/or incorrect translational start/stop sites, so knowing the sequences of the proteins expressed by an organism will help to improve gene predictions.

The proteogenomic analysis carried out in this study addresses some of these challenges by improving the characterization of predicted proteins from the annotated *N. americanus* genome. Overall, we identified 121 peptides that map to intergenic regions in the first draft genome sequence for *N. americanus*. Peptides that map to intergenic regions are highly significant as they can lead to identification of novel protein-coding genes or corrections of pre-existing models (31). To investigate whether these peptides are likely to be new genes we checked for any potential ORFs where the peptides map. Thirty-two (32) of the peptides identified mapped to alternative ORFs than those described in the current gene model. While these peptides map to known coding regions, they highlight out-of-frame ORFs which is likely to, once correctly annotated, result in an entirely different protein. Of the total newly identified peptides, 39 of them mapped to introns. Peptides mapping to introns can lead to identification of novel splice isoforms or amendments in gene structure. Peptides mapping to exon extensions and N-terminal extensions were less abundant, with 10 and 4 peptides respectively. These groups of peptides suggest a possible correction in reading frame or an incorrect start site annotation.

The decrease in gene number seen in this study is in line with other genome annotations. For example, the *Schistosoma mansoni* genome was originally thought to encode 11,809 genes, however further annotation has reduced this number to 10,772 (60). Given that this is the first re-annotation of the *N. americanus* genome since the original draft was published, a substantial decrease in gene number was to be expected, and 15,728 genes is comparable with the predicted gene numbers of other nematode genomes (61). Despite being a parasitic nematode, *N. americanus* has almost 5,000 fewer genes than its free-living relative *C. elegans* (https://parasite.wormbase.org/).

Of particular importance in the characterization of parasite-specific genes is the presence of a signal peptide. Of the secreted ES proteins from *N. americanus*, 51% were predicted to have a signal peptide; this is also in agreement with ES proteomes from other parasitic nematodes (15, 19, 52). The presence of extracellular proteins without predicted signal peptides in the ES products of *N. americanus* could be due to one of three reasons: (a) the protein is secreted via an alternative pathway, including release of parasite exosomes (62-65) or non-classical secretory signals; (b) the lack of full-length RNA transcript sequence to confirm gene model accuracy resulted in an error in the predicted sequence (i.e. truncation or ORF shift); (c) the pre-set D-cutoff threshold of 0.33 in SignalP results in false-negative predictions.

Helminth secretomes represent the molecular host-parasite interface (10), and provide useful insights into the biological strategies employed by these parasites to ensure longevity inside their respective hosts (10). At this interface, ES products have been implicated in numerous roles from initial penetration/invasion and feeding to host immune regulation (10). Obtaining sufficient ES products to generate the proteome described in this study was time consuming due to the difficulty in culturing sufficient quantities of parasite material in hamsters. For this reason, human infection with *N. americanus* is frequently modelled using other hookworms and related nematodes that survive in rodents or larger animals, including *Ancylostoma sp., H. polygyrus* and *N. brasiliensis.* The protein family analysis of *N. americanus* ES products revealed a diverse number of known and unknown domains (391 domains total), which attests to the many biological functions of ES proteins as well as to the lack of information on these proteomes. The ES products of the three comparator species used here had similar protein family profiles with 458 (*A. caninum*), 434 (*H. polygyrus*) and 628 (*N. brasiliensis*) unique domains present. To assess the overall usefulness of these models, a similarity analysis was carried out on their ES products. This analysis relates relative protein sequence similarities in a plot where each of the identified *N. americanus* ES proteins is compared to the ES proteome of the comparator species (19, 51). The majority of the *N. americanus* ES proteins (173/198), including 51/54 SCP/TAPS proteins, had homologs in the ES products of all three comparators, highlighting the relevance and usefulness of all three models. A total of 25 proteins did not have similarity to ES proteins from the other 3 nematodes analyzed. Since *A. caninum*, *H. polygyrus* and *N. brasiliensis* are animal parasites, these 25 proteins might have evolved to such an extent that they target human-specific pathways. Two unique proteins of interest are the aspartyl protease NAME_01848 (PF00026) and the zinc metalloprotease NAME_05081 (PF01546). Metalloproteases and aspartyl proteases play crucial roles in host tissue penetration and parasite feeding, and as such, proteolytic enzymes from helminths may be of particular interest as vaccine and/or drug targets (66, 67).

The most abundantly represented protein family (54/198; 27%) in *N. americanus* ES products was proteins containing a single or double SCP/TAPS domain (PF00188). This finding aligns with previous work that highlighted the abundance of this protein family in nematode ES products in particular (15, 68). For instance, a total of 45, 90 and 25 SCP/TAPS proteins were found in the ES products from *N. brasiliensis* (45/313; 14%), *A. caninum* (90/315; 29%) and *H. polygyrus* respectively (25/374; 12%). Interestingly, this family of proteins is also abundant in the extracellular vesicles secreted by different nematodes (64, 65). Given that SCP/TAPS proteins from *N. brasiliensis* are almost exclusively secreted by the adult developmental stage (19), these proteins are likely coordinating specific roles in the gastrointestinal tract of the host. In fact, it has also been shown that SCP/TAPS are overrepresented at the transcript level in *N. americanus* adult worms (11). While relatively little functional information is available for SCP/TAPS proteins, neutrophil inhibitory factor (NIF), an SCP/TAPS protein in the ES of *A. caninum*, was reported to abrogate neutrophil adhesion to the endothelium (69). However, a *N. americanus* homolog of this protein was not detected in the current study, despite the presence of a NIF-encoding gene in the draft genome (11). SCP/TAPS proteins are thought to play numerous and diverse roles at the host-parasite interface, from defense mechanisms, normal body formation and lifespan (54). The diverse nature of *N. americanus* SCP/TAPS sequences is evidenced by their phylogenetic relationships (Figure 5A and B). Blast analyses presented in the Circos plot (Figure 4A) reveal varying degrees of sequence homology to SCP/TAPS proteins from *A. caninum*, *H. polygyrus* or *N. brasiliensis*. These SCP/TAPS proteins should be further explored to understand their roles in *N. americanus*-human host interactions. Given the limited availability of information regarding the function of helminth SCP/TAPS proteins in general, it was unsurprising that GO analyses revealed no known molecular function or biological process for 32/54 of the SCP/TAPS from *N. americanus* ES products, and more studies should be performed to characterize the properties of this intriguing family of proteins.

Despite the lack of functional information on the SCP/TAPS proteins in parasitic helminths, of the species we compared in this study, *N. americanus* proteins were generally most similar to those from *A. caninum*, which could simply be a reflection of the phylogenetic similarity between the two species. (Figure 5A and 5B). The trees generated in this study highlight strong clade-specific similarities between SCP/TAPS in the ES products of the compared species. In support of this, the vast majority of the SCP/TAPS proteins came from the four clade V species, while *A. suum*, *T. muris* and *T. canis* had only 2, 5 and 2 SCP/TAPS proteins respectively. The clustering of *A. caninum* with *N. americanus* and *H. polygyrus* with *N. brasiliensis* supports the notion of host-specific roles for SCP/TAPS. Another trend across both single and double domain trees was the diversity of evolution between SCP/TAPS within a single species. For example, the single-domain tree included a sub-clade of 7 *N. americanus*-only sequences indicating an important human-specific role for this evolutionary cluster. This compares well with a number of other sub-clades which included proteins from the four predominant species. These SCP/TAPS proteins are more likely to share a common function, potentially in host-infection or parasite development.

The phylogenetic analysis strongly attests to the preferred use of *A. caninum* for studying hookworm molecular biology and ES products in general (70). This finding is reinforced by the Circos plot of SCP/TAPS (Figure 4A). Not only was there a greater number of SCP/TAPS homologs in the ES products of *A. caninum* but these proteins also had relatively higher blast scores (denoted by link ribbon thickness) and higher percent identity scores (denoted by ribbon darkness). This type of analysis can prove useful since it also reveals which species to consider for investigating a specific *Necator* SCP/TAPS protein. For example, NAME_13850 has significant sequence homology to a SCP/TAPS protein from each of the compared species; however, the species with the highest sequence homology is *A. caninun* (ANCCAN_19759), making this protein the most relevant to study as a model for NAME_13850. Prior to updating the genome, all three species of nematode used in this comparison had longer average gene sequence lengths than the *N. americanus* SCP/TAPS sequences. In the previous annotation, the average *N. americanus* SCP/TAPS sequence length was 244 predicted amino acids, compared with 355 residues in the updated genome. This was likely due to some of the sequences being truncated, yielding similar results to the published *H. polygyrus* proteome (52).

The Circos plot representing *N. americanus* proteases and homologous proteins from the three comparator species (Figure 4B) provides insight into the high degree of sequence similarity. Unlike the SCP/TAPS Circos analysis, all the *N. americanus* ES proteases had homologs with high similarity in the compared species (average 50% identity between *N. americanus* and comparator species protease). This finding supports the notion of using any one of these three parasites to study *N. americanus* proteases in general. We identified 8 aspartyl, 6 cysteine, 9 metallo and 3 serine proteases in the ES products of *N. americanus*. As the adult stage of *N. americanus* feeds on blood, the high abundance of aspartyl proteases was expected (25, 71). Yet when compared to other species, the human hookworm ES products included more of these proteases. Aspartyl proteases play a fundamental role in the digestion of host hemoglobin and have also been implicated in skin penetration, feeding, and host tissue degradation (72, 73). The finding that *N. americanus* has more of this mechanistic class of proteases than the other nematodes assessed here is likely due to split gene models and/or may be a true gene family expansion. Due to the vital role that aspartyl proteases play in parasite feeding, *Na*-APR-1 an *N. americanus* aspartyl protease, was targeted as a vaccine candidate (74). While we were unable to detect *Na*-APR-1 in the current ES proteome - probably because it is anchored to the gut epithelium (75) - 9 other aspartyl proteases were detected which could potentially be targeted as novel vaccine candidates.

Cysteine proteases, particularly the group belonging to the papain superfamily, are common in nematodes (76). They have been specifically described for their proteolytic activity against hemoglobin, antibodies and fibrinogen in the *N. americanus* lifecycle (77). Similarly, another study highlighted the importance of four cysteine proteases that were upregulated in the *N. americanus* transition from free-living larvae to blood-feeding adult worm, indicating that these proteins are likely to be important for nutrient acquisition (78). Metalloproteases - particularly the astacins - identified in this study are most likely important for larval and adult migration and invasion through human host tissue (79). In support of this, astacin metalloproteases were found to be upregulated in *N. brasiliensis* larvae when compared with adult stage parasites (19). Interestingly, *N. americanus* metalloproteases were reported to inhibit eosinophil recruitment through the cleavage of eotaxin, a potent eosinophil chemoattractant (23). The least abundant family of proteases in the adult *N. americanus* ES products were the serine proteases. Relatively little is known about these proteases from *N. americanus* specifically; however, a serine protease from the whipworm *T. muris* is involved in degradation of the mucus barrier to facilitate feeding (80). Due to the importance of these various proteases in parasite feeding, infection, migration and defense, they represent potential targets for chemotherapies and vaccines to limit infection (81).

In the current study, we have provided the first proteogenomic analysis of a helminth parasite, resulting in a more accurate genome annotation. While the above annotations are valuable additions to the current genome, further improvement requires access to substantially more parasitic material including adult stage parasites which are difficult to obtain. Furthermore, we have carried out the first proteomic analysis of the ES products of the human hookworm *N. americanus*. The results presented herein offer significant insight into the validity of these model species while also highlighting differences between these important parasites.

Acknowledgements

This work was supported by a program grant from the National Health and Medical Research Council (NHMRC) [program grant number 1037304] and a Senior Principal Research fellowship from NHMRC to AL (1117504). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Work performed at Washington University School of Medicine was supported by NIH-NIAID grant AI081803 to MM. JL was supported by an Australian Postgraduate Award and by the Australian Institute of Tropical Health and Medicine.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010669.

To access the data, please use the below login details.

Username: reviewer49665@ebi.ac.uk

Password: fybW0mgJ

The newly annotated, improved gene models were used in subsequent proteomic analysis of ES products, and are publicly available on Nematode.net.

References

1. Ngui, R., Lim, Y. A., Traub, R., Mahmud, R., and Mistam, M. S. (2012) Epidemiological and genetic data supporting the transmission of *Ancylostoma ceylanicum* among human and domestic animals. *PLoS Negl Trop Dis* 6, e1522

2. Loukas, A., Hotez, P. J., Diemert, D., Yazdanbakhsh, M., McCarthy, J. S., Correa-Oliveira, R., Croese, J., and Bethony, J. M. (2016) Hookworm infection. *Nat Rev Dis Primers* 2, 16088

3. Hotez, P., Brooker, S., Bethony, J., Bottazzi, M. E., Loukas, A., and Xiao, S. (2004) Hookworm Infection. *N Engl J Med* 351, 799-807

4. Brooker, S., Bethony, J., and Hotez, P. J. (2004) Human Hookworm Infection in the 21st Century. *Adv Parasitol* 58, 197-288

5. Christian, P., Khatry, S. K., and West, K. P. (2004) Antenatal anthelmintic treatment, birthweight, and infant survival in rural Nepal. *Lancet* 364, 981-983

6. Brooker, S., Hotez, P. J., and Bundy, D. A. (2008) Hookworm-related anaemia among pregnant women: a systematic review. *PLoS Negl Trop Dis* 2, e291

7. Murray, C. J. L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J. A., Abdalla, S., Aboyans, V., Abraham, J., Ackerman, I., Aggarwal, R., Ahn, S. Y., Ali, M. K., AlMazroa, M. A., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Bahalim, A. N., Barker-Collo, S., Barrero, L. H., Bartels, D. H., Basáñez, M.-G., Baxter, A., Bell, M. L., Benjamin, E. J., Bennett, D., Bernabé, E., Bhalla, K., Bhandari, B., Bikbov, B., Abdulhak, A. B., Birbeck, G., Black, J. A., Blencowe, H., Blore, J. D., Blyth, F., Bolliger, I., Bonaventure, A., Boufous, S., Bourne, R., Boussinesq, M., Braithwaite, T., Brayne, C., Bridgett, L., Brooker, S., Brooks, P., Brugha, T. S., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Buckle, G., Budke, C. M., Burch, M., Burney, P., Burstein, R.,

Calabria, B., Campbell, B., Canter, C. E., Carabin, H., Carapetis, J., Carmona, L., Cella, C., Charlson, F., Chen, H., Cheng, A. T.-A., Chou, D., Chugh, S. S., Coffeng, L. E., Colan, S. D., Colquhoun, S., Colson, K. E., Condon, J., Connor, M. D., Cooper, L. T., Corriere, M., Cortinovis, M., de Vaccaro, K. C., Couser, W., Cowie, B. C., Criqui, M. H., Cross, M., Dabhadkar, K. C., Dahiya, M., Dahodwala, N., Damsere-Derry, J., Danaei, G., Davis, A., Leo, D. D., Degenhardt, L., Dellavalle, R., Delossantos, A., Denenberg, J., Derrett, S., Des Jarlais, D. C., Dharmaratne, S. D., Dherani, M., Diaz-Torne, C., Dolk, H., Dorsey, E. R., Driscoll, T., Duber, H., Ebel, B., Edmond, K., Elbaz, A., Ali, S. E., Erskine, H., Erwin, P. J., Espindola, P., Ewoigbokhan, S. E., Farzadfar, F., Feigin, V., Felson, D. T., Ferrari, A., Ferri, C. P., Fèvre, E. M., Finucane, M. M., Flaxman, S., Flood, L., Foreman, K., Forouzanfar, M. H., Fowkes, F. G. R., Fransen, M., Freeman, M. K., Gabbe, B. J., Gabriel, S. E., Gakidou, E., Ganatra, H. A., Garcia, B., Gaspari, F., Gillum, R. F., Gmel, G., Gonzalez-Medina, D., Gosselin, R., Grainger, R., Grant, B., Groeger, J., Guillemin, F., Gunnell, D., Gupta, R., Haagsma, J., Hagan, H., Halasa, Y. A., Hall, W., Haring, D., Haro, J. M., Harrison, J. E., Havmoeller, R., Hay, R. J., Higashi, H., Hill, C., Hoen, B., Hoffman, H., Hotez, P. J., Hoy, D., Huang, J. J., Ibeanusi, S. E., Jacobsen, K. H., James, S. L., Jarvis, D., Jasrasaria, R., Jayaraman, S., Johns, N., Jonas, J. B., Karthikeyan, G., Kassebaum, N., Kawakami, N., Keren, A., Khoo, J.-P., King, C. H., Knowlton, L. M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Laden, F., Lalloo, R., Laslett, L. L., Lathlean, T., Leasher, J. L., Lee, Y. Y., Leigh, J., Levinson, D., Lim, S. S., Limb, E., Lin, J. K., Lipnick, M., Lipshultz, S. E., Liu, W., Loane, M., Ohno, S. L., Lyons, R., Mabweijano, J., MacIntyre, M. F., Malekzadeh, R., Mallinger, L., Manivannan, S., Marcenes, W., March, L., Margolis, D. J., Marks, G. B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B. M., McAnulty, J. H., McDermott, M. M., McGill, N., McGrath, J., Medina-Mora, M. E., Meltzer, M., Memish, Z. A., Mensah, G. A., Merriman, T.

R., Meyer, A.-C., Miglioli, V., Miller, M., Miller, T. R., Mitchell, P. B., Mock, C., Mocumbi, A. O., Moffitt, T. E., Mokdad, A. A., Monasta, L., Montico, M., Moradi-Lakeh, M., Moran, A., Morawska, L., Mori, R., Murdoch, M. E., Mwaniki, M. K., Naidoo, K., Nair, M. N., Naldi, L., Narayan, K. M. V., Nelson, P. K., Nelson, R. G., Nevitt, M. C., Newton, C. R., Nolte, S., Norman, P., Norman, R., O'Donnell, M., O'Hanlon, S., Olives, C., Omer, S. B., Ortblad, K., Osborne, R., Ozgediz, D., Page, A., Pahari, B., Pandian, J. D., Rivero, A. P., Patten, S. B., Pearce, N., Padilla, R. P., Perez-Ruiz, F., Perico, N., Pesudovs, K., Phillips, D., Phillips, M. R., Pierce, K., Pion, S., Polanczyk, G. V., Polinder, S., Pope Iii, C. A., Popova, S., Porrini, E., Pourmalek, F., Prince, M., Pullan, R. L., Ramaiah, K. D., Ranganathan, D., Razavi, H., Regan, M., Rehm, J. T., Rein, D. B., Remuzzi, G., Richardson, K., Rivara, F. P., Roberts, T., Robinson, C., De Leòn, F. R., Ronfani, L., Room, R., Rosenfeld, L. C., Rushton, L., Sacco, R. L., Saha, S., Sampson, U., Sanchez-Riera, L., Sanman, E., Schwebel, D. C., Scott, J. G., Segui-Gomez, M., Shahraz, S., Shepard, D. S., Shin, H., Shivakoti, R., Silberberg, D., Singh, D., Singh, G. M., Singh, J. A., Singleton, J., Sleet, D. A., Sliwa, K., Smith, E., Smith, J. L., Stapelberg, N. J. C., Steer, A., Steiner, T., Stolk, W. A., Stovner, L. J., Sudfeld, C., Syed, S., Tamburlini, G., Tavakkoli, M., Taylor, H. R., Taylor, J. A., Taylor, W. J., Thomas, B., Thomson, W. M., Thurston, G. D., Tleyjeh, I. M., Tonelli, M., Towbin, J. A., Truelsen, T., Tsilimbaris, M. K., Ubeda, C., Undurraga, E. A., van der Werf, M. J., van Os, J., Vavilala, M. S., Venketasubramanian, N., Wang, M., Wang, W., Watt, K., Weatherall, D. J., Weinstock, M. A., Weintraub, R., Weisskopf, M. G., Weissman, M. M., White, R. A., Whiteford, H., Wiebe, N., Wiersma, S. T., Wilkinson, J. D., Williams, H. C., Williams, S. R. M., Witt, E., Wolfe, F., Woolf, A. D., Wulf, S., Yeh, P.-H., Zaidi, A. K. M., Zheng, Z.-J., Zonies, D., and Lopez, A. D. (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2197-2223

8. Hotez, P. (2008) Hookworm and poverty. *Ann N Y Acad Sci* 1136, 38-44

9. Hotez, P. J., Bethony, J., Bottazzi, M. E., Brooker, S., and Buss, P. (2005) Hookworm: "the great infection of mankind". *PLoS Med* 2, e67

10. Hewitson, J. P., Grainger, J. R., and Maizels, R. M. (2009) Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol* 167, 1-11

11. Tang, Y. T., Gao, X., Rosa, B. A., Abubucker, S., Hallsworth-Pepin, K., Martin, J., Tyagi, R., Heizer, E., Zhang, X., Bhonagiri-Palsikar, V., Minx, P., Warren, W. C., Wang, Q., Zhan, B., Hotez, P. J., Sternberg, P. W., Dougall, A., Gaze, S. T., Mulvenna, J., Sotillo, J., Ranganathan, S., Rabelo, E. M., Wilson, R. K., Felgner, P. L., Bethony, J., Hawdon, J. M., Gasser, R. B., Loukas, A., and Mitreva, M. (2014) Genome of the human hookworm *Necator americanus*. *Nature Genet* 46, 261-269

12. Navarro, S., Pickering, D. A., Ferreira, I. B., Jones, L., Ryan, S., Troy, S., Leech, A., Hotez, P. J., Zhan, B., Laha, T., Prentice, R., Sparwasser, T., Croese, J., Engwerda, C. R., Upham, J. W., Julia, V., Giacomin, P. R., and Loukas, A. (2016) Hookworm recombinant protein promotes regulatory T cell responses that suppress experimental asthma. *Sci Transl Med* 8, 362ra143

13. Hotez, P. J., Bethony, J. M., Diemert, D. J., Pearson, M., and Loukas, A. (2010) Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat Rev Microbiol* 8, 814-826

14. Cancado, G. G., Fiuza, J. A., de Paiva, N. C., Lemos Lde, C., Ricci, N. D., Gazzinelli-Guimaraes, P. H., Martins, V. G., Bartholomeu, D. C., Negrao-Correa, D. A., Carneiro, C. M., and

Fujiwara, R. T. (2011) Hookworm products ameliorate dextran sodium sulfate-induced colitis in BALB/c mice. *Inflamm Bowel Dis* 17, 2275-2286

15. Mulvenna, J., Hamilton, B., Nagaraj, S. H., Smyth, D., Loukas, A., and Gorman, J. J. (2009) Proteomics analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, *Ancylostoma caninum*. *Mol Cell Proteomics* 8, 109-121

16. Hotez, P. J., Beaumier, C. M., Gillespie, P. M., Strych, U., Hayward, T., and Bottazzi, M. E. (2016) Advancing a vaccine to prevent hookworm disease and anemia. *Vaccine* 34, 3001-3005

17. Diemert, D. J., Freire, J., Valente, V., Fraga, C. G., Talles, F., Grahek, S., Campbell, D., Jariwala, A., Periago, M. V., Enk, M., Gazzinelli, M. F., Bottazzi, M. E., Hamilton, R., Brelsford, J., Yakovleva, A., Li, G., Peng, J., Correa-Oliveira, R., Hotez, P., and Bethony, J. (2017) Safety and immunogenicity of the Na-GST-1 hookworm vaccine in Brazilian and American adults. *PLoS Negl Trop Dis* 11, e0005574

18. Hunt, V. L., Tsai, I. J., Coghlan, A., Reid, A. J., Holroyd, N., Foth, B. J., Tracey, A., Cotton, J. A., Stanley, E. J., Beasley, H., Bennett, H. M., Brooks, K., Harsha, B., Kajitani, R., Kulkarni, A., Harbecke, D., Nagayasu, E., Nichol, S., Ogura, Y., Quail, M. A., Randle, N., Xia, D., Brattig, N. W., Soblik, H., Ribeiro, D. M., Sanchez-Flores, A., Hayashi, T., Itoh, T., Denver, D. R., Grant, W., Stoltzfus, J. D., Lok, J. B., Murayama, H., Wastling, J., Streit, A., Kikuchi, T., Viney, M., and Berriman, M. (2016) The genomic basis of parasitism in the Strongyloides clade of nematodes. *Nature Genet* 48, 299-307

19. Sotillo, J., Sanchez-Flores, A., Cantacessi, C., Harcus, Y., Pickering, D., Bouchery, T., Camberis, M., Tang, S., Giacomin, P., Mulvenna, J., Mitreva, M., Berriman, M., LeGros, G., Maizels, R., and Loukas, A. (2014) Secreted proteomes of different developmental stages of the gastrointestinal nematode *Nippostrongylus brasiliensis*. *Mol Cell Proteomics* 13, 2736-2751

20. Morante, T., Shepherd, C., Constantinoiu, C., Loukas, A., and Sotillo, J. (2017) Revisiting the *Ancylostoma caninum* secretome provides new information on hookworm-host interactions. *Proteomics* 17

21. Hsieh, G. C. F., Loukas, A., Wahl, A. M., Bhatia, M., Wang, Y., Williamson, A. L., Kehn, K. W., Maruyama, H., Hotez, P. J., Leitenberg, D., Bethony, J., and Constant, S. L. (2004) A secreted protein from the human hookworm *Necator americanus* binds selectively to NK cells and induces IFN- production. *J Immunol* 173, 2699-2704

22. Winter, J., Davies, O., Brown, A., Garnett, M., Stolnik, S., and Pritchard, D. (2005) The assessment of hookworm calreticulin as a potential vaccine for necatoriasis. *Parasite Immunol* 27, 139–146

23. Culley, F. J., Brown, A., Conroy, D. M., Sabroe, I., Pritchard, D. I., and Williams, T. J. (2000) Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action *in vitro* and *in vivo*. *J Immunol* 165, 6447-6453

24. Kumar, S., and Pritchard, D. I. (1992) Secretion of metalloproteases by living infective larvae of *Necator americanus*. *J Parasitol* 78, 917-919

25. Ranjit, N., Zhan, B., Hamilton, B., Stenzel, D., Lowther, J., Pearson, M., Gorman, J., Hotez, P., and Loukas, A. (2009) Proteolytic degradation of hemoglobin in the intestine of the human hookworm *Necator americanus*. *J Infect Dis* 199, 904-912

26. Cantacessi, C., Mitreva, M., Jex, A. R., Young, N. D., Campbell, B. E., Hall, R. S., Doyle, M. A., Ralph, S. A., Rabelo, E. M., Ranganathan, S., Sternberg, P. W., Loukas, A., and Gasser, R. B. (2010) Massively parallel sequencing and analysis of the *Necator americanus* transcriptome. *PLoS Negl Trop Dis* 4, e684

27. Wang, Z., Abubucker, S., Martin, J., Wilson, R. K., Hawdon, J., and Mitreva, M. (2010) Characterizing *Ancylostoma caninum* transcriptome and exploring nematode parasitic adaptation. *BMC Genomics* 11, 307-307

28. Schwarz, E. M., Hu, Y., Antoshechkin, I., Miller, M. M., Sternberg, P. W., and Aroian, R. V. (2015) The genome and transcriptome of the zoonotic hookworm *Ancylostoma ceylanicum* identify infection-specific gene families. *Nat Genet* 47, 416-422

29. Palevich, N., Britton, C., Kamenetzky, L., Mitreva, M., de Moraes Mourão, M., Bennuru, S., Quack, T., Scholte, L. L. S., Tyagi, R., and Slatko, B. E. (2018) Tackling hypotheticals in helminth genomes. *Trends Parasitol* 34, 179-183

30. Borchert, N., Dieterich, C., Krug, K., Schutz, W., Jung, S., Nordheim, A., Sommer, R. J., and Macek, B. (2010) Proteogenomics of *Pristionchus pacificus* reveals distinct proteome structure of nematode models. *Genome Res* 20, 837-846

31. Nesvizhskii, A. I. (2014) Proteogenomics: concepts, applications and computational strategies. *Nat Methods* 11, 1114-1125

32. Holt, C., and Yandell, M. (2011) MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects. *BMC Bioinformatics* 12, 491

33. Bao, W., Kojima, K. K., and Kohany, O. (2015) Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mob DNA* 6, 11

34. Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114-2120

35. Kim, D., Langmead, B., and Salzberg, S. L. (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 12, 357-360

36. Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T. C., Mendell, J. T., and Salzberg, S. L. (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* 33, 290-295

37. Hoff, K. J., Lange, S., Lomsadze, A., Borodovsky, M., and Stanke, M. (2016) BRAKER1: Unsupervised RNA-Seq-Based genome annotation with GeneMark-ET and AUGUSTUS. *Bioinformatics* 32, 767-769

38. The UniProt, C. (2017) UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 45, D158-D169

39. Howe, K. L., Bolt, B. J., Shafie, M., Kersey, P., and Berriman, M. (2017) WormBase ParaSite - a comprehensive resource for helminth genomics. *Mol Biochem Parasitol* 215, 2-10

40. Campbell, M. S., Holt, C., Moore, B., and Yandell, M. (2014) Genome annotation and curation ising MAKER and MAKER-P. *Curr Protoc Bioinformatics* 48, 4 11 11-39

41. Lomsadze, A., Burns, P. D., and Borodovsky, M. (2014) Integration of mapped RNA-Seq reads into automatic training of eukaryotic gene finding algorithm. *Nucleic Acids Res* 42, e119

42. Stanke, M., Diekhans, M., Baertsch, R., and Haussler, D. (2008) Using native and syntenically mapped cDNA alignments to improve *de novo* gene finding. *Bioinformatics* 24, 637-644

43. Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S. Y., Lopez, R., and Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236-1240

44. Campbell, M. S., Law, M., Holt, C., Stein, J. C., Moghe, G. D., Hufnagel, D. E., Lei, J., Achawanantakun, R., Jiao, D., Lawrence, C. J., Ware, D., Shiu, S. H., Childs, K. L., Sun, Y., Jiang,

N., and Yandell, M. (2014) MAKER-P: a tool kit for the rapid creation, management, and quality control of plant genome annotations. *Plant Physiol* 164, 513-524

45. Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015) BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31, 3210-3212

46. Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674-3676

47. Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* 2, 953-971

48. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305, 567-580

49. Supek, F., Bosnjak, M., Skunca, N., and Smuc, T. (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* 6, e21800

50. Conway, J. R., Lex, A., and Gehlenborg, N. (2017) UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* 33, 2938-2940

51. Parkinson, J., and Blaxter, M. (2003) SimiTri--visualizing similarity relationships for groups of sequences. *Bioinformatics* 19, 390-395

52. Hewitson, J. P., Harcus, Y., Murray, J., van Agtmaal, M., Filbey, K. J., Grainger, J. R., Bridgett, S., Blaxter, M. L., Ashton, P. D., Ashford, D. A., Curwen, R. S., Wilson, R. A., Dowle, A. A., and Maizels, R. M. (2011) Proteomic analysis of secretory products from the model

gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of venom allergen-like (VAL) proteins. *J Proteomics* 74, 1573-1594

53. Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S. J., and Marra, M. A. (2009) Circos: an information aesthetic for comparative genomics. *Genome Res* 19, 1639-1645

54. Cantacessi, C., Campbell, B. E., Visser, A., Geldhof, P., Nolan, M. J., Nisbet, A. J., Matthews, J. B., Loukas, A., Hofmann, A., Otranto, D., Sternberg, P. W., and Gasser, R. B. (2009) A portrait of the "SCP/TAPS" proteins of eukaryotes--developing a framework for fundamental research and biotechnological outcomes. *Biotechnol Adv* 27, 376-388

55. Letunic, I., and Bork, P. (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127-128

56. Martin, J., Rosa, B. A., Ozersky, P., Hallsworth-Pepin, K., Zhang, X., Bhonagiri-Palsikar, V., Tyagi, R., Wang, Q., Choi, Y.-J., Gao, X., McNulty, S. N., Brindley, P. J., and Mitreva, M. (2015) Helminth.net: expansions to Nematode.net and an introduction to Trematode.net. *Nucleic Acids Res* 43, D698-D706

57. Martin, J., Tyagi, R., Rosa, B. A., and Mitreva, M. (2018) A multi-omics database for parasitic nematodes and trematodes. *Methods Mol Biol* 1757, 371-397

58. Jaffe, J. D., Berg, H. C., and Church, G. M. (2004) Proteogenomic mapping as a complementary method to perform genome annotation. *Proteomics* 4, 59-77

59. Sotillo, J., Toledo, R., Mulvenna, J., and Loukas, A. (2017) Exploiting helminth-host interactomes through big data. *Trends Parasitol* 33, 875-888

60. Protasio, A. V., Tsai, I. J., Babbage, A., Nichol, S., Hunt, M., Aslett, M. A., De Silva, N., Velarde, G. S., Anderson, T. J., Clark, R. C., Davidson, C., Dillon, G. P., Holroyd, N. E., LoVerde,

P. T., Lloyd, C., McQuillan, J., Oliveira, G., Otto, T. D., Parker-Manuel, S. J., Quail, M. A., Wilson, R. A., Zerlotini, A., Dunne, D. W., and Berriman, M. (2012) A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. *PLoS Negl Trop Dis* 6, e1455

61. Zarowiecki, M., and Berriman, M. (2015) What helminth genomes have taught us about parasite evolution. *Parasitology* 142, S85-S97

62. Coakley, G., Buck, A. H., and Maizels, R. M. (2016) Host parasite communications—Messages from helminths for the immune system: Parasite communication and cell-cell interactions. *Mol Biochem Parasitol* 208, 33-40

63. Marcilla, A., Martin-Jaular, L., Trelis, M., de Menezes-Neto, A., Osuna, A., Bernal, D., Fernandez-Becerra, C., Almeida, I. C., and del Portillo, H. A. (2014) Extracellular vesicles in parasitic diseases. *J Extracell Vesicles* 3, 10.3402/jev.v3403.25040

64. Eichenberger, R. M., Ryan, S., Jones, L., Buitrago, G., Polster, R., Montes de Oca, M., Zuvelek, J., Giacomin, P. R., Dent, L. A., Engwerda, C. R., Field, M. A., Sotillo, J., and Loukas, A. (2018) Hookworm secreted extracellular vesicles interact with host cells and prevent inducible colitis in mice. *Front Immunol* 9

65. Eichenberger, R. M., Talukder, M. H., Field, M. A., Wangchuk, P., Giacomin, P., Loukas, A., and Sotillo, J. (2018) Characterization of *Trichuris muris* secreted proteins and extracellular vesicles provides new insights into host–parasite communication. *J Extracell Vesicles* 7, 1428004

66. Knox, D. (2011) Proteases in blood-feeding nematodes and their potential as vaccine candidates. *Adv Exp Med Biol* 712, 155-176

67. Abdulla, M. H., Lim, K. C., Sajid, M., McKerrow, J. H., and Caffrey, C. R. (2007) *Schistosomiasis mansoni*: novel chemotherapy using a cysteine protease inhibitor. *PLoS Med* 4, e14

68. Hewitson, J. P., Ivens, A. C., Harcus, Y., Filbey, K. J., McSorley, H. J., Murray, J., Bridgett, S., Ashford, D., Dowle, A. A., and Maizels, R. M. (2013) Secretion of protective antigens by tissue-stage nematode larvae revealed by proteomic analysis and vaccination-induced sterile immunity. *PLoS Pathog* 9, e1003492

69. Lo, S. K., Rahman, A., Xu, N., Zhou, M. Y., Nagpala, P., Jaffe, H. A., and Malik, A. B. (1999) Neutrophil inhibitory factor abrogates neutrophil adhesion by blockade of CD11a and CD11b beta(2) integrins. *Mol Pharmacol* 56, 926-932

70. Shepherd, C., Wangchuk, P., and Loukas, A. (2018) Of dogs and hookworms: man's best friend and his parasites as a model for translational biomedical research. *Parasit Vectors* 11, 59

71. Ranjit, N., Jones, M. K., Stenzel, D. J., Gasser, R. B., and Loukas, A. (2006) A survey of the intestinal transcriptomes of the hookworms, *Necator americanus* and *Ancylostoma caninum*, using tissues isolated by laser microdissection microscopy. *Int J Parasitol* 36, 701-710

72. Williamson, A. L., Brindley, P. J., Abbenante, G., Prociv, P., Berry, C., Girdwood, K., Pritchard, D. I., Fairlie, D. P., Hotez, P. J., Dalton, J. P., and Loukas, A. (2002) Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. *FASEB J* 16, 1458-1460

73. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc Natl Acad Sci USA* 97, 1456-1460

74. Hotez, P. J., Diemert, D., Bacon, K. M., Beaumier, C., Bethony, J. M., Bottazzi, M. E., Brooker, S., Couto, A. R., da Silva Freire, M., Homma, A., Lee, B. Y., Loukas, A., Loblack, M., Morel, C. M., Oliveira, R. C., and Russell, P. K. (2013) The Human Hookworm Vaccine. *Vaccine* 31, B227-B232

75. Loukas, A., Bethony, J. M., Mendez, S., Fujiwara, R. T., Goud, G. N., Ranjit, N., Zhan, B., Jones, K., Bottazzi, M. E., and Hotez, P. J. (2005) Vaccination with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. *PLoS Med* 2, e295

76. Ono, Y., and Sorimachi, H. (2012) Calpains — An elaborate proteolytic system. *Biochim. Biophys. Acta, Proteins Proteomics* 1824, 224-236

77. Baig, S., Damian, R. T., and Peterson, D. S. (2002) A novel cathepsin B active site motif is shared by helminth bloodfeeders. *Exp Parasitol* 101, 83-89

78. Ranjit, N., Zhan, B., Stenzel, D. J., Mulvenna, J., Fujiwara, R., Hotez, P. J., and Loukas, A. (2008) A family of cathepsin B cysteine proteases expressed in the gut of the human hookworm, *Necator americanus*. *Mol Biochem Parasitol* 160, 90-99

79. Williamson, A. L., Lustigman, S., Oksov, Y., Deumic, V., Plieskatt, J., Mendez, S., Zhan, B., Bottazzi, M. E., Hotez, P. J., and Loukas, A. (2006) *Ancylostoma caninum* MTP-1, an astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. *Infect Immun* 74, 961-967

80. Hasnain, S. Z., McGuckin, M. A., Grencis, R. K., and Thornton, D. J. (2012) Serine protease(s) secreted by the nematode *Trichuris muris* degrade the mucus barrier. *PLoS Negl Trop Dis* 6, e1856

81. Mendez, S., Zhan, B., Goud, G., Ghosh, K., Dobardzic, A., Wu, W., Liu, S., Deumic, V., Dobardzic, R., Liu, Y., Bethony, J., and Hotez, P. J. (2005) Effect of combining the larval antigens Ancylostoma secreted protein 2 (ASP-2) and metalloprotease 1 (MTP-1) in protecting hamsters against hookworm infection and disease caused by *Ancylostoma ceylanicum*. *Vaccine* 23, 3123-3130

Table 1: Comparison of original and updated genome annotations

	Original annotation	Updated annotation
Number of genes	19,153	15,728
Number of exons	122,849	148,780
Number of introns	103,696	133,052
Number of CDS	19,153	15,728
Overlapping genes	395	2,424
Contained genes	2	386
Total gene length (bp)	82,090,364	126,651,725
Total exon length (bp)	15,470,227	24,553,753
Total intron length (bp)	66,827,529	102,364,076
Total CDS length (bp)	15,461,420	17,683,227
Mean gene length (bp)	4,286	8,053
Mean exon length (bp)	126	165
Mean intron length (bp)	644	769
Mean CDS length (bp)	807	1,124
% of genome covered by genes	33.6	51.9
% of genome covered by CDS	6.3	7.2
Mean exons per mRNA	6.4	9.5
Mean introns per mRNA	5.4	8.5
Complete BUSCOs	82.84%	88.45%
Fragmented BUSCOs	12.87%	8.91%
Missing BUSCOs	4.29%	2.64%

Table 2: Top 30 most abundant proteins in the ES products of *Necator americanus*

		MASCOT score		iProphet probabilty		NP				emPAI		
Accession	Blast2GO Description	Ingel	Offgel	Ingel	Offgel	Ingel	Offgel	SP	TD	Ingel	Offgel	Domain
NAME_05596	Hypothetical protein	19496	15287	1	1	11	11	N	0	867.19	308.05	No conserved domains
NAME_07794	Hypothetical protein	21210	39596	1	1	18	22	Y	0	47.88	199.86	Single SCP
NAME_11917	SCP	35077	43195	1	1	18	31	N	0	11.87	68.4	Double SCP
NAME_13724	Hypothetical protein	7701	23099	1	1	5	7	N	0	7.06	64.55	No conserved domains
NAME_15197	SCP	9446	12866	1	1	6	7	N	0	9.55	50.11	No conserved domains
NAME_14329	SCP	10911	15142	1	1	10	15	N	0	14.34	43.25	Single SCP
NAME_11146	SCP	21158	29296	1	1	24	27	Y	0	17.03	22.85	Double SCP
NAME_10941	Hypothetical protein	983	3870	1	1	3	8	Y	0	2.6	33.23	Single SCP
NAME_09098	SCP	6908	47379	1	1	13	25	Y	0	3.27	31.87	Double SCP
NAME_05595	SCP-like protein, partial	0	23369	0	1	0	10	Y	0	0	26.61	Single SCP
NAME_11145	SCP	19923	25558	1	1	18	22	N	0	6.35	16.62	Double SCP
NAME_02907	Glu Leu Phe Val dimerization	6645	6095	1	1	20	22	N	0	6.82	7.63	Single ELFV_dehydrog_N
NAME_10752	Nematode fatty acid retinoid	12416	3051	1	1	11	8	Y	0	9.76	4.65	Single Gp-FAR-1
NAME_05865	Globin	3566	5667	1	1	6	10	N	0	2.63	9.67	Single Globin
NAME_15231	SCP	117	1844	0	1	3	6	N	0	1.75	9.66	Single SCP
NAME_05592	Hypothetical protein	0	4150	0	1	0	4	Y	0	0	11.02	Single SCP
NAME_13132	Ferritin	1808	2833	1	1	7	8	N	0	3.39	7.31	Single Ferritin-like
NAME_05794	Hypothetical protein	2681	2271	1	1	7	9	N	0	5.18	5.21	No conserved domains
NAME_13809	SCP	8287	14385	1	1	7	9	Y	0	3.99	5.92	Single SCP
NAME_07942	Copper zinc superoxide dismutase	4241	4469	1	1	4	8	N	0	1.41	6.25	Single Copper/zinc superoxide
NAME_09181	Hypothetical protein	27774	68153	1	1	41	50	N	0	3.17	4.43	No conserved domains
NAME_10294	SCP	6557	11684	1	1	7	9	Y	0	2.58	4.8	Single SCP
NAME_02523	Histidine operon leader	23085	24957	1	1	66	63	Y	0	3.68	3.3	Many NPA
NAME_13379	Flavodoxin	0	1089	0.9985	0	0	4	N	0	0	6.89	No conserved domains
NAME_02090	SCP	3916	12638	1	1	8	14	Y	0	1.35	5.47	Double SCP

NAME_01069	SCP	3816	11201	1	1	10	12	Y	0	1.91	4.63	Double SCP
NAME_12806	SCP	8094	8066	1	1	6	8	Y	0	1.62	4.42	Single SCP
NAME_02548	Globin	887	1473	1	1	4	6	N	0	3.01	3.03	Single Globin
NAME_05396	SCP	1339	5930	1	1	4	8	Y	0	1.48	4.13	Single SCP
NAME_13850	SCP	3510	4006	1	0.9933	7	8	Y	0	1.97	3.07	Single SCP

Table legends

Table 1: Summary of the updated genome annotation of *Necator americanus*. CDS – coding DNA sequence.

Table 2: Top 30 most abundant proteins identified with in-gel and OFF-GEL fractionation and ranked using summed exponential modified protein abundance index (emPAI) score. Blast2GO was used to obtain descriptions of each protein. Abbreviations key: NP – number of significant peptides; SP – signal peptide; TD – transmembrane domain; Mascot scores >28 indicate identity or extensive homology ($p < 0.05$).

Figure legends

Figure 1: Depiction of the proteogenomic process as well as the types and numbers of peptide corrections identified.

Figure 2: (A) UpSetR plot displaying the number of each gene ontology (GO) term categories (biological process, molecular function and/or cellular component) available (Blast2GO) for adult *Necator americanus* excretory/secretory (ES) proteins. Proteins with no available GO terms are broken down in a pie chart into 'SCP/TAPS' proteins 'other'. **(B)** Top 10 most abundant protein families in the ES products of adult *N. americanus*. **(C)** Biological processes of adult *N. americanus* ES proteins ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database. **(D)** Molecular functions of adult *N. americanus* ES proteins ranked by nodescore (Blast2GO) plotted using REViGO.

Figure 3: ES products from *Ancylostoma caninum*, *Heligmosomoides polygyrus*, *and Nippostrongylus brasiliensis* were compared with the excretory/secretory proteome of *Necator americanus* and displayed in a Simitri plot. SCP/TAPS are represented by an orange square, proteases by a teal-colored triangle and any other protein by a grey circle. Points on the diagram triangle represent sequences which only had similarity to the labelled species. Points along the edges of the triangle are sequences which had similarity to two of the three species (given at the respective ends of the edge). Any sequence in the middle area of the triangle represents a sequence with similarity to all three compared species.

Figure 4: SCP/TAPS proteins in the excretory/secretory products of *Necator americanus* are most closely related to SCP/TAPS proteins in the ES of *Ancylostoma caninum*. SCP/TAPS **(A)** and protease **(B)** protein names are displayed in a circle with *N. americanus* (purple), *A. caninum* (blue), *Heligmosomoides polygyrus* (pink), and *Nippostrongylus brasiliensis* (green). Ribbon thickness is relative to the maximum score obtained in the BLAST search while darker ribbons denote higher sequence percent identity. The corresponding bars provide relative sequence length of each protein. Respective protease mechanistic classes: aspartic (ASP), cysteine (CYS), metallo (MET) or serine (SER).

Figure 5: Phylogenetic relationships of **(A)** single-domain and **(B)** double-domain SCP/TAPS proteins determined with MUSCLE alignment software. PhyML was used for a maximum-likelihood phylogenetic analysis and results were visualized with The Interactive Tree of Life (iTOF) online phylogeny tool. *Necator americanus* sequences are highlighted in purple with comparator species each denoted by a different color (see key).

Figure 1

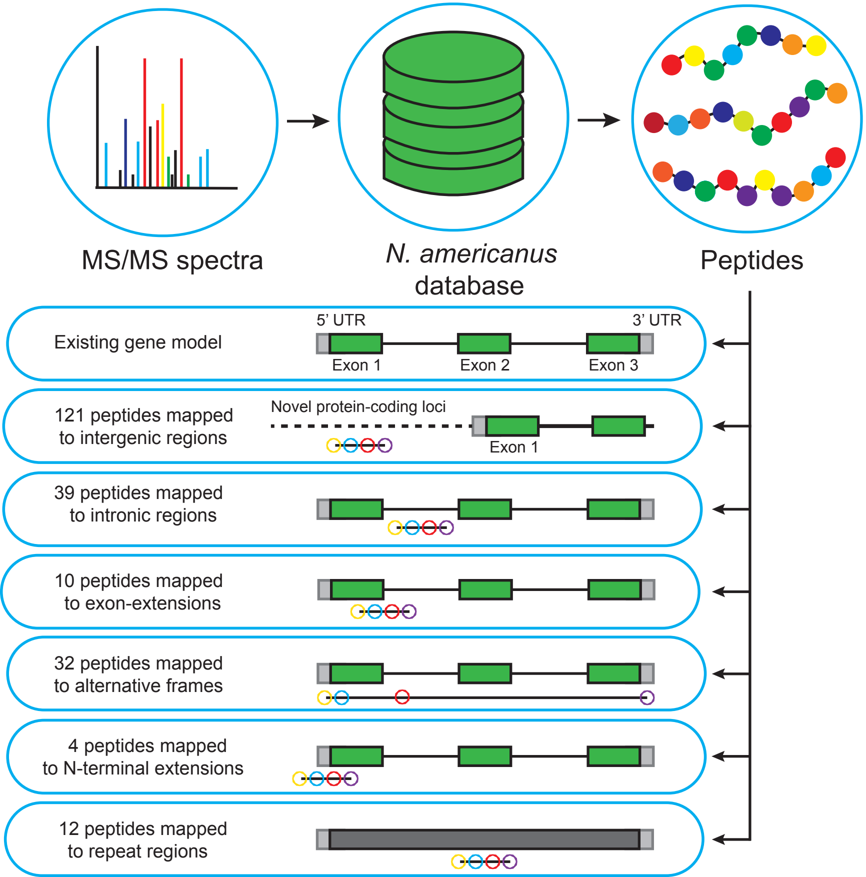
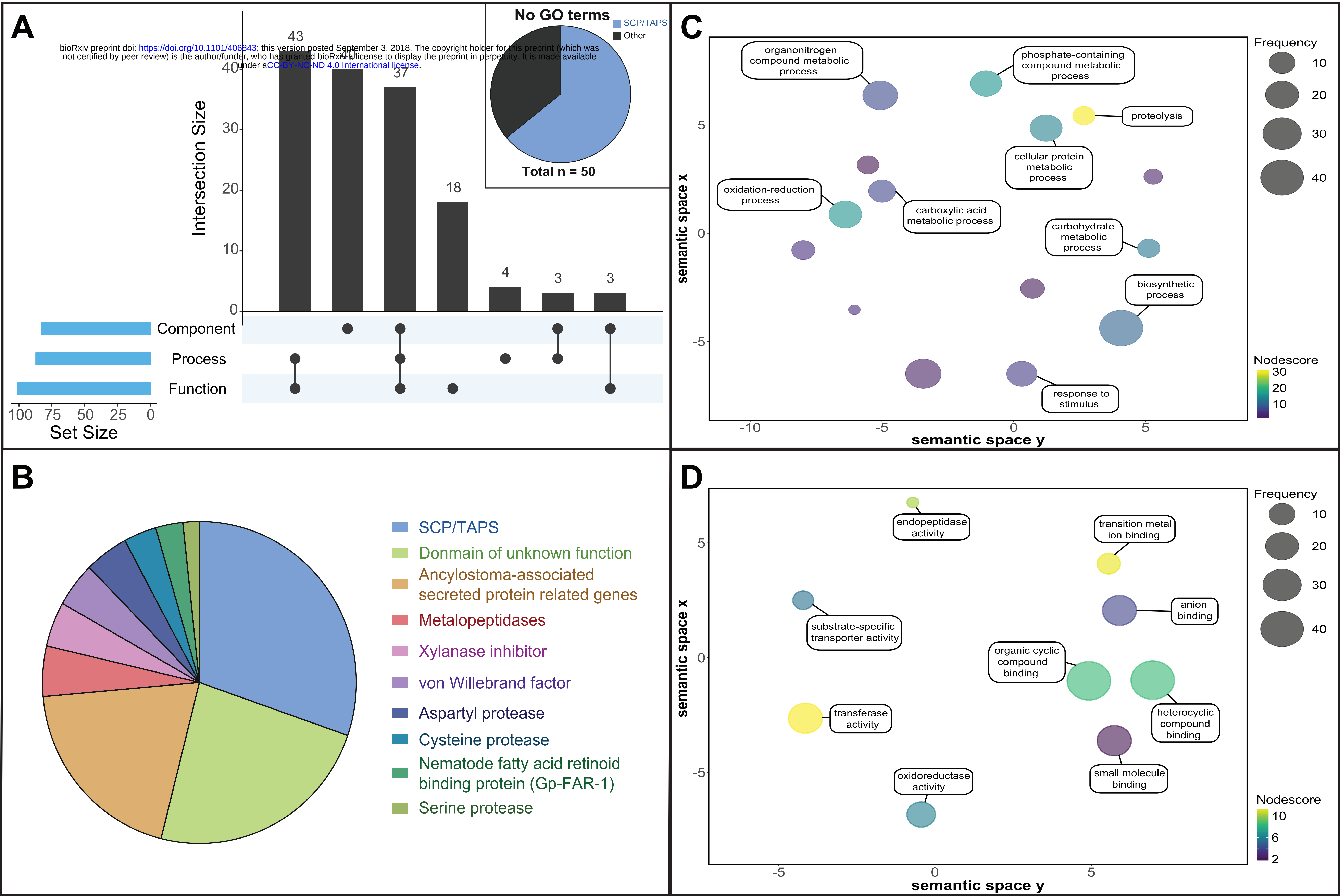

MS/MS spectra
N. americanus
database
Peptides
Existing gene model
5' UTR
3' UTR
Exon 1
Exon 2
Exon 3
121 peptides mapped
to intergenic regions
Novel protein-coding loci
Exon 1
39 peptides mapped
to intronic regions
10 peptides mapped
to exon-extensions
32 peptides mapped
to alternative frames
4 peptides mapped
to N-terminal extensions
12 peptides mapped
to repeat regions

Figure 2


A
Intersection Size
0
10
20
30
40
43
40
37
18
4
3
3
No GO terms
SCP/TAPS
Other
Total n = 50
Component
Process
Function
100 75 50 25 0
Set Size
B
SCP/TAPS
Donmain of unknown function
Ancylostoma-associated secreted protein related genes
Metalopeptidases
Xylanase inhibitor
von Willebrand factor
Aspartyl protease
Cysteine protease
Nematode fatty acid retinoid binding protein (Gp-FAR-1)
Serine protease
C
organonitrogen compound metabolic process
phosphate-containing compound metabolic process
proteolysis
cellular protein metabolic process
oxidation-reduction process
carboxylic acid metabolic process
carbohydrate metabolic process
biosynthetic process
response to stimulus
semantic space x
semantic space y
5
0
-5
-10
-5
0
5
Frequency
10
20
30
40
Nodescore
30
20
10
D
endopeptidase activity
transition metal ion binding
anion binding
substrate-specific transporter activity
organic cyclic compound binding
heterocyclic compound binding
transferase activity
small molecule binding
oxidoreductase activity
semantic space x
semantic space y
5
0
-5
-5
0
5
Frequency
10
20
30
40
Nodescore
10
6
2

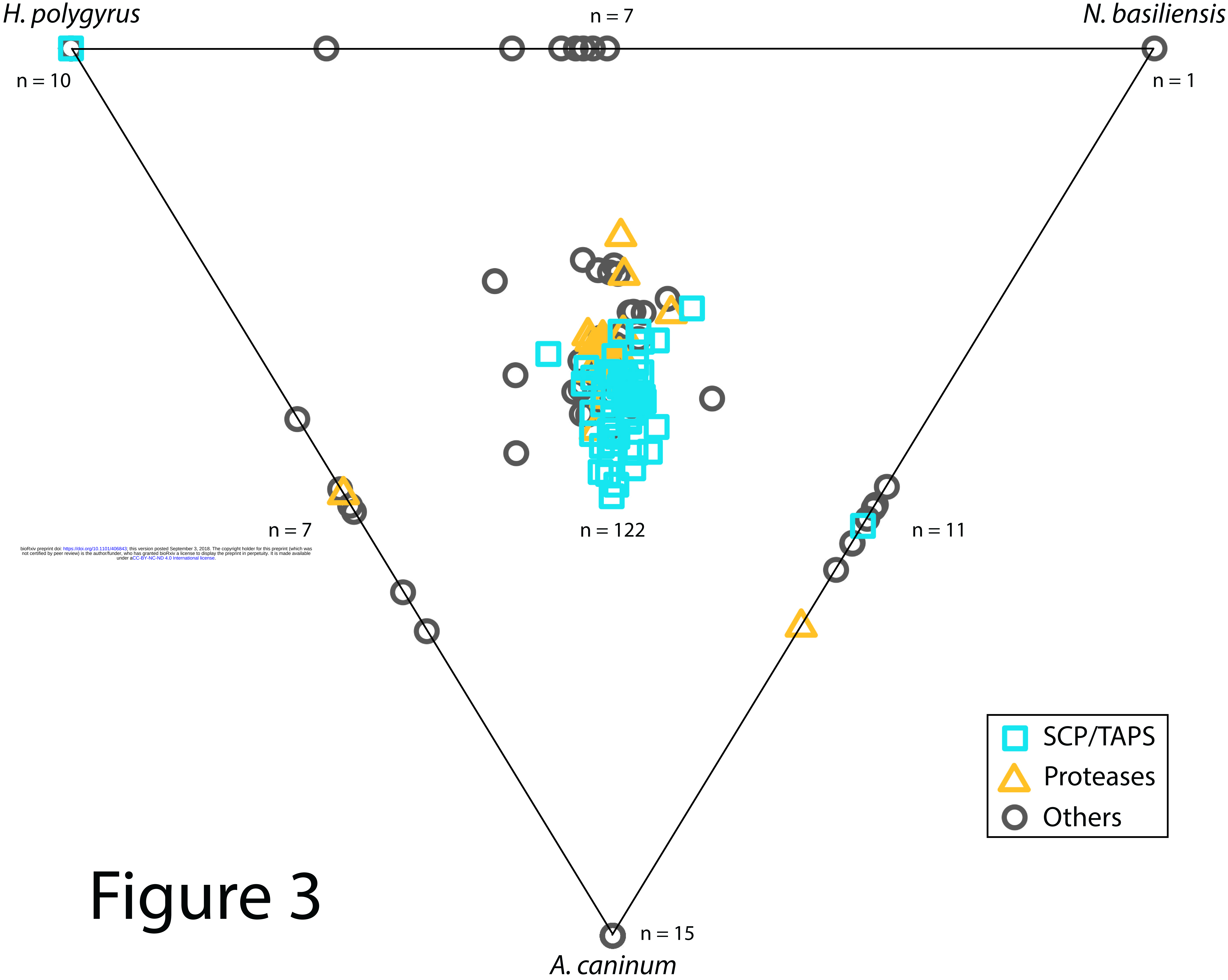

H. polygyrus
n = 7
N. basiliensis
n = 10
n = 1
n = 7
n = 122
n = 11
□ SCP/TAPS
△ Proteases
○ Others
n = 15
A. caninum

Figure 3

Figure 4A

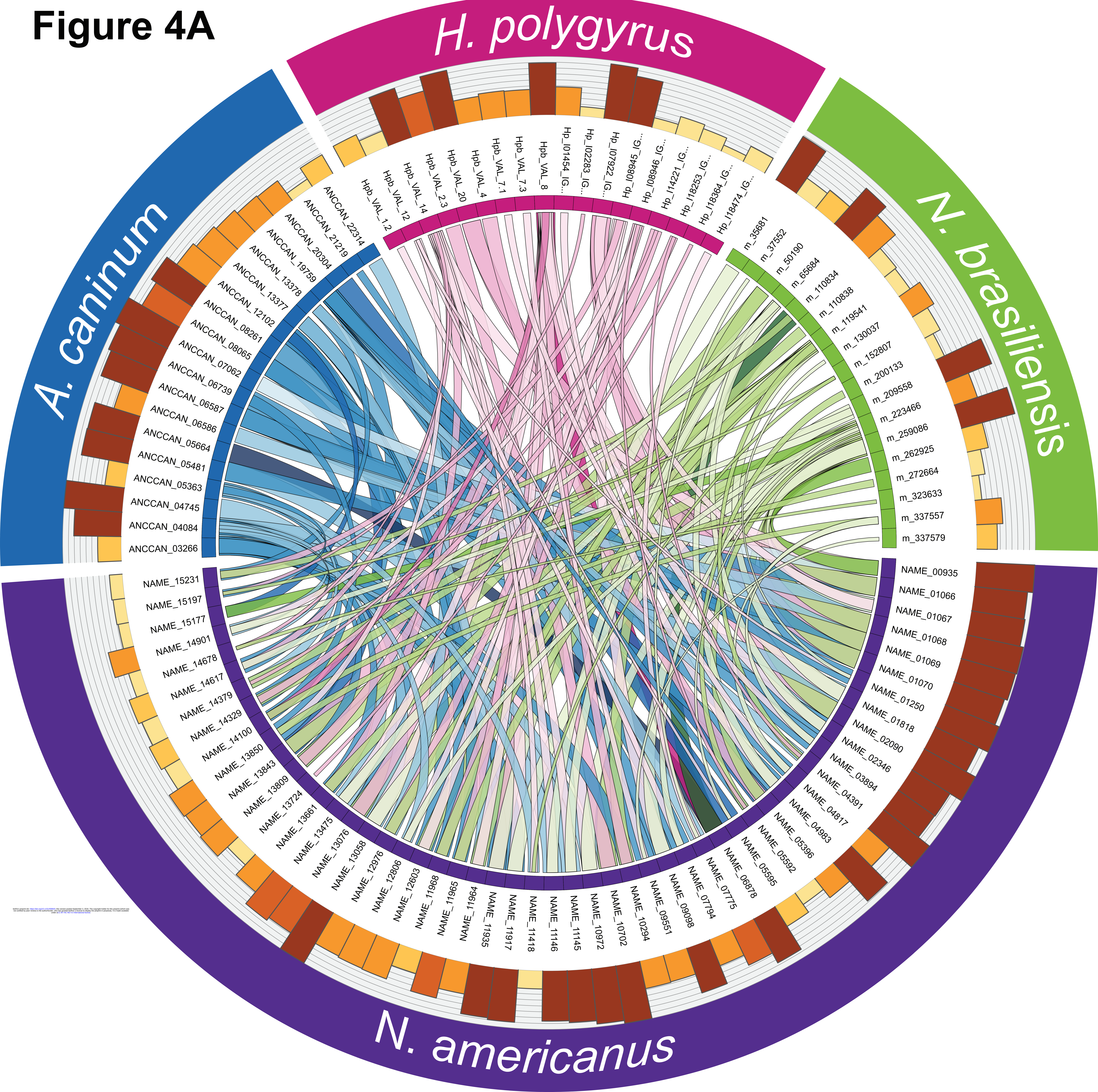

H. polygyrus
N. brasiliensis
A. caninum
N. americanus
Hpb_VAL_1.2
Hpb_VAL_12
Hpb_VAL_14
Hpb_VAL_2.3
Hpb_VAL_20
Hpb_VAL_4
Hpb_VAL_7.1
Hpb_VAL_7.3
Hpb_VAL_8
Hp_I01454_IG...
Hp_I02283_IG...
Hp_I07922_IG...
Hp_I08945_IG...
Hp_I08946_IG...
Hp_I14221_IG...
Hp_I18253_IG...
Hp_I18364_IG...
Hp_I18474_IG...
m_35681
m_37552
m_50190
m_65684
m_110834
m_110838
m_119541
m_130037
m_152807
m_200133
m_209558
m_223466
m_259086
m_262925
m_272664
m_323633
m_337557
m_337579
NAME_00935
NAME_01066
NAME_01067
NAME_01068
NAME_01069
NAME_01070
NAME_01250
NAME_01818
NAME_02090
NAME_02346
NAME_03894
NAME_04391
NAME_04817
NAME_04983
NAME_05396
NAME_05592
NAME_05595
NAME_06878
NAME_07775
NAME_07794
NAME_09098
NAME_09551
NAME_10294
NAME_10702
NAME_10972
NAME_11145
NAME_11146
NAME_11418
NAME_11917
NAME_11935
NAME_11964
NAME_11965
NAME_11968
NAME_12603
NAME_12806
NAME_12976
NAME_13058
NAME_13076
NAME_13475
NAME_13661
NAME_13724
NAME_13809
NAME_13843
NAME_13850
NAME_14100
NAME_14329
NAME_14379
NAME_14617
NAME_14678
NAME_14901
NAME_15177
NAME_15197
NAME_15231
ANCCAN_03266
ANCCAN_04084
ANCCAN_04745
ANCCAN_05363
ANCCAN_05481
ANCCAN_05664
ANCCAN_06586
ANCCAN_06587
ANCCAN_06739
ANCCAN_07062
ANCCAN_08065
ANCCAN_08261
ANCCAN_12102
ANCCAN_13377
ANCCAN_13378
ANCCAN_19759
ANCCAN_20304
ANCCAN_21219
ANCCAN_22314

Figure 4B

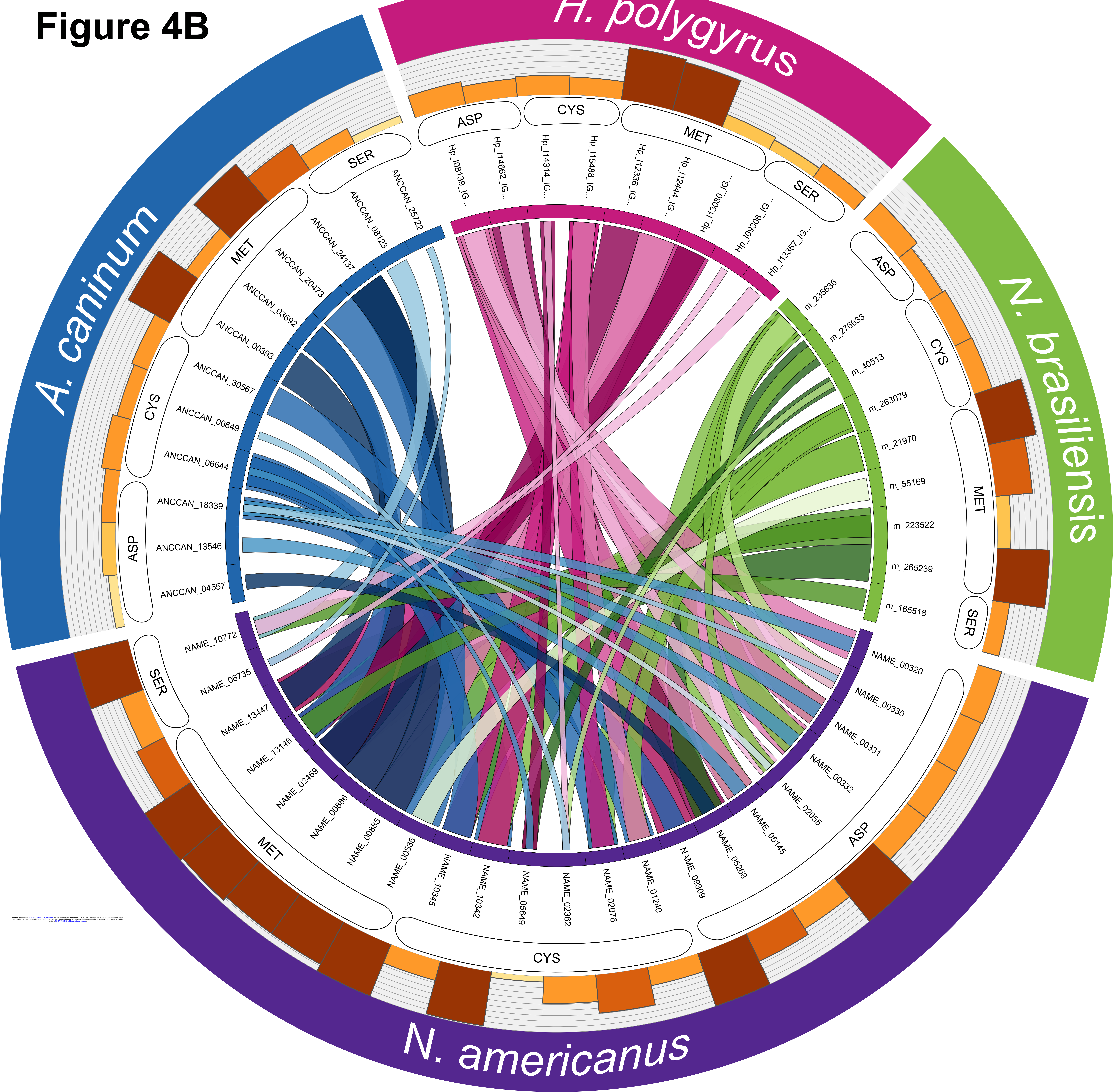

H. polygyrus
ASP
CYS
MET
SER
Hp_I08139_IG...
Hp_I14662_IG...
Hp_I14314_IG...
Hp_I15488_IG...
Hp_I12336_IG...
Hp_I12444_IG...
Hp_I13080_IG...
Hp_I09306_IG...
Hp_I13357_IG...
N. brasiliensis
ASP
CYS
MET
SER
m_235636
m_276633
m_40513
m_263079
m_21970
m_55169
m_223522
m_265239
m_165518
N. americanus
ASP
CYS
MET
SER
NAME_00320
NAME_00330
NAME_00331
NAME_00332
NAME_02055
NAME_05145
NAME_05268
NAME_09309
NAME_01240
NAME_02076
NAME_02362
NAME_05649
NAME_10342
NAME_10345
NAME_00535
NAME_00885
NAME_00886
NAME_02469
NAME_13146
NAME_13447
NAME_06735
NAME_10772
A. caninum
ASP
CYS
MET
SER
ANCCAN_04557
ANCCAN_13546
ANCCAN_18339
ANCCAN_06644
ANCCAN_06649
ANCCAN_30567
ANCCAN_00393
ANCCAN_03692
ANCCAN_20473
ANCCAN_24137
ANCCAN_08123
ANCCAN_25722

Figure 5A

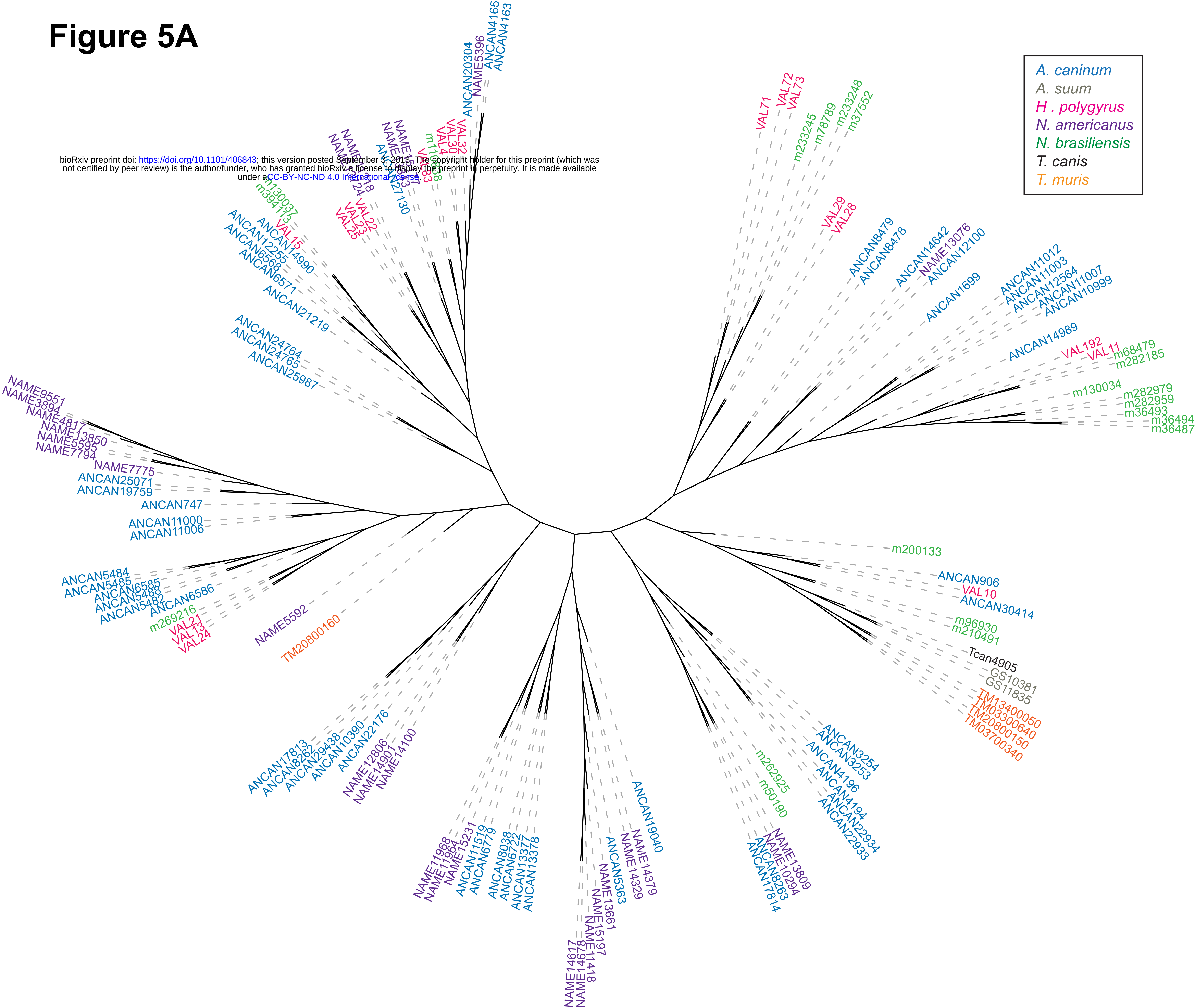
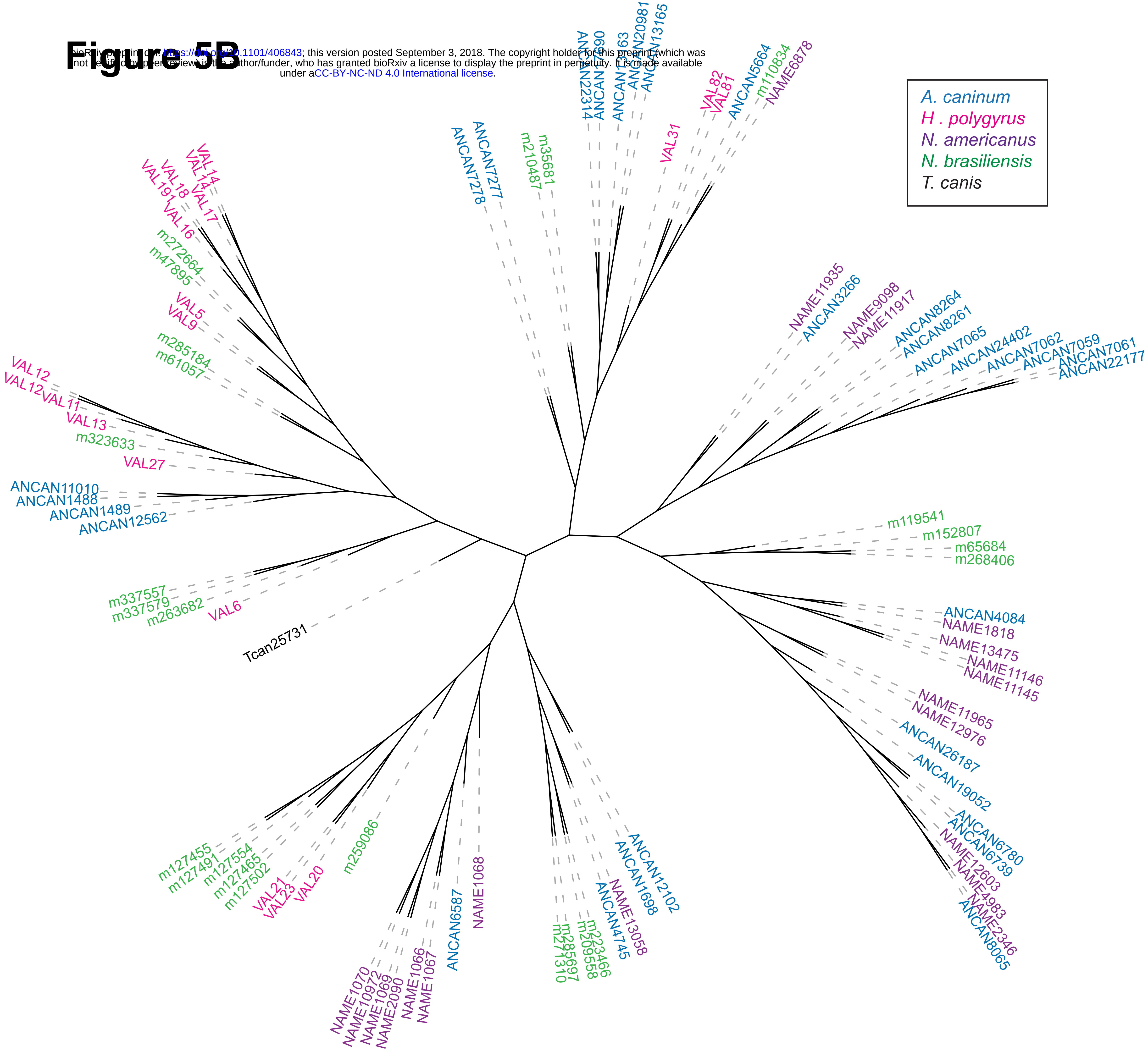

A. caninum
A. suum
H . polygyrus
N. americanus
N. brasiliensis
T. canis
T. muris
ANCAN4165
ANCAN4163
NAME5396
ANCAN20304
VAL32
VAL30
VAL4
m110838
VAL83
ANCAN27130
m130037
m39413
VAL15
VAL22
VAL23
VAL25
ANCAN14990
ANCAN12255
ANCAN6568
ANCAN6571
ANCAN21219
ANCAN24764
ANCAN24765
ANCAN25987
NAME9551
NAME3894
NAME4817
NAME13850
NAME5595
NAME7794
NAME7775
ANCAN25071
ANCAN19759
ANCAN747
ANCAN11000
ANCAN11006
ANCAN5484
ANCAN5485
ANCAN6585
ANCAN5488
ANCAN5482
ANCAN6586
m269216
VAL21
VAL13
VAL24
NAME5592
TM20800160
ANCAN17813
ANCAN8262
ANCAN29438
ANCAN10390
ANCAN22176
NAME12806
NAME14901
NAME14100
NAME11968
NAME11964
NAME15231
ANCAN11519
ANCAN6779
ANCAN8038
ANCAN6722
ANCAN13377
ANCAN13378
NAME14617
NAME14678
NAME11418
NAME15197
NAME13661
ANCAN5363
NAME14329
NAME14379
ANCAN19040
NAME13809
NAME10294
ANCAN8263
ANCAN17814
m262925
m50190
ANCAN3254
ANCAN3253
ANCAN4196
ANCAN4194
ANCAN22934
ANCAN22933
TM13400050
TM03300640
TM20800150
TM03700340
GS10381
GS11835
Tcan4905
m96930
m210491
ANCAN906
VAL10
ANCAN30414
m200133
m36487
m36494
m36493
m282959
m282979
m130034
m68479
m282185
VAL192
VAL11
ANCAN14989
ANCAN11012
ANCAN11003
ANCAN12564
ANCAN11007
ANCAN10999
ANCAN1699
ANCAN14642
NAME13076
ANCAN12100
ANCAN8479
ANCAN8478
VAL29
VAL28
m233245
m78789
m233248
m37552
VAL71
VAL72
VAL73

Figure 5B


A. caninum
H . polygyrus
N. americanus
N. brasiliensis
T. canis
ANCAN7278
ANCAN7277
m210487
m35681
ANCAN22314
ANCAN17690
ANCAN13163
ANCAN20981
ANCAN13165
VAL31
VAL82
VAL81
ANCAN5664
m110834
NAME6878
NAME11935
ANCAN3266
NAME9098
NAME11917
ANCAN8264
ANCAN8261
ANCAN7065
ANCAN24402
ANCAN7062
ANCAN7059
ANCAN7061
ANCAN22177
m119541
m152807
m65684
m268406
ANCAN4084
NAME1818
NAME13475
NAME11146
NAME11145
NAME11965
NAME12976
ANCAN26187
ANCAN19052
ANCAN6780
ANCAN6739
NAME12603
NAME4983
NAME2346
ANCAN8065
ANCAN12102
ANCAN1698
NAME13058
ANCAN4745
m223466
m209558
m285697
m271310
NAME1068
ANCAN6587
NAME1067
NAME1066
NAME2090
NAME1069
NAME10972
NAME1070
m259086
VAL20
VAL23
VAL21
m127502
m127465
m127554
m127491
m127455
Tcan25731
VAL6
m263682
m337579
m337557
ANCAN12562
ANCAN1489
ANCAN1488
ANCAN11010
VAL27
m323633
VAL13
VAL11
VAL12
VAL12
m61057
m285184
VAL9
VAL5
m47895
m272664
VAL16
VAL191
VAL18
VAL17
VAL14
VAL14