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Genomics and Transcriptomics of Antarctic Nematodes Reveal Drivers of Life History Evolution and Genome Evolution

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A dissertation submitted to the faculty of
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ABSTRACT

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Elemental stoichiometry defines a critical understanding of the relationship between nutrient availability and usage throughout different levels of the biological community. We found there is a link between available phosphorus (P), cellular phosphorus, and nematode development as postulated by the growth rate hypothesis (GRH). I predicted that in a P-poor environment, cellular RNA concentrations would be lower than they are in P-rich environment, and thus the 18srRNA expression level will have reduced. To most efficiently regulate the uptake of limited P, I predicted that nematodes in P-poor environments would decrease the number of copies of the 18s rRNA gene in their genome. I measured life history traits as well as rRNA gene expression and gene copy number. We found that elemental stoichiometry predicts evolutionary changes consistent with the Growth Rate Hypothesis. We sequenced and assembled a draft genome of *P. murrayi.* Although we expected to find genes responsible for stress tolerance, we hypothesized that in response to strong selection pressure associated with living in a simplified ecosystem, over time the genome of *P. murrayi* should have undergone significant decay (gene loss) relative to species in ecosystems structured more strongly by biotic interactions. We found significantly fewer genes in *P. murrayi*. To compare patterns of gene expression between two highly divergent Antarctic nematode species, we sequenced and assembled the transcriptomes of *S. lindsayae* and *P. murrayi*. Under laboratory conditions at 4 ˚C, *S. lindsayae* had significantly lower rates of gene expression but expressed a significantly larger number of genes. We speculate that the differences in gene expression are correlated with life history traits (developmental rates) while the differences in the number of genes expressed can be explained by their different genetic systems (*S. lindsayae* is amphimictic, *P. murrayi* is parthenogenic) and the soil environments to which they are adapted. Since we previously showed that differences in available P content can influence the evolution of gene expression via gene copy number, and that this ultimately influences growth rate, we wondered how much of this response is driven by genetics versus how strongly these patterns are driven by temperature. To better understand this, we maintained wild type populations of *P. murrayi* in P-rich and P-poor conditions at 5 ˚C, 10 ˚C and 15˚C in the laboratory for over 40 generations and sequenced the transcriptomes prepared from each treatment group. We found that nutrient levels played an important role in gene expression when the temperature is optimal for *P. murrayi* culturing and that temperature is more important in gene expression when the available P is limited. This work underscores the utility of using principles of elemental stoichiometry coupled with genomic and transcriptomics research tools to make and test predictions about life history evolution. The results of my work also inform inferences about the ways in which nutrient availability also drives the organization of trophic interactions and ultimately ecosystems.

Keywords: Antarctic nematodes, *Caenorhabditis elegans,* genome evolution, growth rate hypothesis, *Plectus murrayi*, *Scottnema lindsayae*, transcriptome

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Chapter 1

An emerging model for studying evolutionary biology in an extreme ecosystem: *Plectus murrayi*

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Abstract

Nematode *Plectus murrayi* is one of the dominant continental organisms only found in Antarctica, and it is merging as a model organism for studying on evolutionary biology in high stresses environment. This study aims to review the current studies of its phylogeny, distribution, life cycle and the development in the laboratory, as well as its freezing and desiccation tolerance on a genetic level. Compared to other Antarctic nematodes, *P. murrayi* prefers relative higher moisture and lower salinity soil habitats, which might be consistent with its broad distribution in more productive habitats of Antarctica. Because the temperature and humidity increasing in Antarctic McMurdo Dry Valleys (MDV), *P. murrayi* may becoming to the dominant continental animal in Antarctic deserts. By researching on *P. murrayi*, we hope to be able to explore new connections between its adaptation and functional genes that are responsible for stress tolerance and stoichiometric constraints (including the GRH and genomic streamlining). Based on studies of short-term and long-term responses of *P. murrayi* to environmental stresses in Antarctica, it is possible to reveal the relationship between the evolution and ecosystem complexity, as well as the role of elemental stoichiometry in shaping communities from more complex ecosystems.

Introduction

Nematodes are widely distributed across all continents, including the polar regions (Bongers and Ferris, 1999). Over 25,000 nematode species have been described (Zhang, 2013) to date and their numerical dominance at various trophic levels makes them essential members in nutrient cycling (Andrassy, 1998). Because of their simple, transparent body structures and relatively shorter lifespans, some nematode species have been studied as model organisms in multiple scientific disciplines, such as genetic engineering, neural science, and developmental biology (Andrassy, 1998; Wharton and Raymond, 2015).

Plectus, a common nematode genus that occurs in both aquatic and terrestrial habitats, contains over 70 described species (Holovachov, 2004). It is one of the first nematode taxa described from Antarctica in 1904, and is also one of the most common and most taxonomically challenging nematodes (Holovachov, 2004). Taxonomic identity of *Plectus* populations in Antarctica have differed widely among various nematologists, with *Plectus antarcticus* recognized as the most "complicated" species (Holovachov, 2004). Regarding *Plectus*, *P. Antarctic*a occurs in maritime Antarctica and *Plectus murrayi* in continental Antarctica (Andrassy, 1998; Andrássy and Gibson, 2006; Kito and Ohyama, 2008; Maslen and Convey, 2006). Nevertheless, because of it is difficult to identify for non-specialists and limitation of sampling, many of the taxonomic resolutions remain unresolved (Adams et al., 2006).

In general, *Plectus* have a cuticle with transverse striae and scattered, fine bristles. Different species in *Plectus* could have distinctive head shapes offset by the slight constriction. Their tails contain three caudal glands, each of which opens at the tip through a terminal duct. *Plectus* nematode is one of the most broadly distributed and abundant terrestrial nematodes in Antarctica and inhabits both semi-aquatic and terrestrial biotypes (Andrassy, 1998). *P. murrayi*

has a multiple year life cycle characterized by the slower production of eggs relative to other nematodes. In the laboratory, it takes approximately 6-8 weeks at 15 ˚C to complete a life cycle from egg to adult (Adhikari et al., 2010a). Moreover, its active period in Antarctica, including development and reproduction, is limited to only a short time of the year because of the short growing supported season, which makes it easier to be cultured and studied within the limited period (Wall, 2005). It is not difficult to distinguish *P. murrayi* due to their ventrally curved tail (Heyns, 1995) and it is widespread in the more productive and moist soils in Antarctica (Adams et al., 2014a). Despite its relatively wide distribution and abundance, male *P. murrayi* occurs very rarely, and when they are found, they are malfunctional (Lahl et al., 2003). Like other *Plectus* nematodes, parthenogenesis is the dominant mode of reproduction of *P. murrayi*. This subsequent lack of heterozygosity makes its genome relatively easy to assemble and annotate (Andrassy, 1985).

P. murrayi has an important and unique phylogenetic position relative to the origin of the Secernentean radiation, and it is an excellent target for research on genome evolutionary events (Blaxter et al., 1998; Holterman et al., 2006). Additionally, its high tolerance to multiple environmental stressors (desiccation, freezing, high concentration of heavy metals, etc.) has also been studied by many researchers (Adhikari et al., 2009b, 2010b; Hendriksen, 1983; Nkem et al., 2005). *Plectus sp.* has been recorded that could survive total dehydration at 0% relative humidity (RH), even in the absence of induction, and it can keep surviving for one minute under 90% of its internal water lost (Wharton et al., 2005). To understand more about *P. murrayi*, isolating and culturing them in the laboratory becomes important, by which sufficient organisms for additional organism-level studies can be taken place. Fortunately, it is not difficult to grow *P. murrayi* in

the laboratory, which makes it possible to supply sufficient organisms for sequencing and experimental set-ups for further studies.

The Antarctic MDVs are among the driest and coldest deserts on the earth, with less than 100 mm of precipitation per year, and a mean annual air temperature of -20˚C. The life-supporting season may be as short as two months throughout the entire year (Doran et al., 2002; Fountain et al., 1999; Priscu et al., 1998). In this extremely water and nutrient limited soil system, the lack of primary producer makes the trophic community in the MDVs more complex. Nematodes, which occupy the top tier of most of the food web in Antarctica (Wall and Viginia, 1999), play an important role in soil nutrient cycling (Barrett et al., 2008). Because of the unique community structure and the functional trophic web in Antarctic soil system, it is critical to study their communities in great detail for revealing their role in soil processes precisely. Because of the extreme cold temperatures, desiccated soils, and cold, dark and long winters (Freckman and Virginia, 1997), they are highly specialized but differ in their suitability to various habitats across the landscape. Accordingly, some researchers (Porazinska and Wall, 2002; Weicht and Moorhead, 2004) have suggested that *P. murrayi* may take several years to complete its life cycle, which ensures that nematode juvenile and adult stages could endure long polar winters. All aboved make *P. murrayi* valuable for studying on nematode survival strategies in the field (Treonis et al., 2000) and in laboratory studies (Adhikari et al., 2010a; Freckman and Virginia, 1997; Treonis and Wall, 2005; Treonis et al., 2000).

Habitat Distribution

The Antarctic ecosystem has a total area of around 4000 km^2 composed of multiple ice-free valleys (Procter, 1984). The limitation imposed on organisms by the extreme physical conditions makes environmental stresses in MDV a significant factor for organism survival.

Because of their poor capability in supporting life, these valleys are thought to have the lowest plant and invertebrate species diversity on earth (Adams et al., 2006). This unique and extreme environment imposes strong environmental selection, such as freezing temperatures, low moisture availability, salt accumulation and constant high winds; only those organisms that are capable of tolerating these stresses have been selected for in Antarctic ecosystem.

Many soil fauna that survives on terrestrial continental Antarctica are endemic (Adams et al., 2014b; Nielsen et al., 2011; Pugh and Convey, 2008) and are distributed with a high degree of heterogeneity (Huiskes et al., 2006). The distribution of Antarctic nematodes is mainly driven by organic nutrients and soil moisture (Powers et al., 1998) and all *Plectus* populations in Victoria Land are found in similar habitats (Ayres et al., 2007), frequently associated with streams having algae (e.g., *Nostoc commune*) and moss (e.g., *Bryum antarcticum*). *P. murrayi* (Andrassy, 2012) has been found and described in continental Antarctica, and it dominates the moss nematode fauna that prefers the wetter and less salty habitats (Nielsen and King, 2015).

Most of *P. murrayi* occur near stream sediments with more productive soil systems (Treonis et al., 1999). Several studies have also reported that soil moisture is another critical driver determining the habitat suitability for *Plectus*,which prefers soil water content of 7-10% (Mouratov et al., 2001). Courtright et al. (2001) also documented *P. murrayi* was more likely to occur in habitats with relatively higher soil moisture and higher soil NH_4-N, NO_3-N, organic C, and organic C/organic N ratios than *Scottnema*. Compared to other *Plectus* species, *P. murrayi* can tolerate a greater diversity of habitats (Adams et al., 2014a; Andrassy, 1998; Andrássy and Gibson, 2006; Wharton and Brown, 2012; Yeates, 1979), which also makes *P. murrayi* an excellent model organism to understanding the genome evolution under different environmental stresses. *P. murrayi* has a broad distribution and can thrive in more different habits. This makes

it an exceptional candidate to study evolutionary adaptations and survival mechanisms to extreme environmental stressors.

To our knowledge, the distribution and environmental functions of *P. murrayi* is based only on studies from a few distinct regions close to research stations and along northern coastal Victoria Land, as well as its environmental function. However, not all ice-free areas in Antarctica have been sampled, its distribution has been measured from only accessible locations. Furthermore, studies throughout the MDV are not systematic with some valleys being studied more heavily (e.g., Taylor Valley) than others (e.g., Wright Valley). These understudied regions require further investigation to determine if they represent new habits and harbor undescribed nematode species (table 1).

Plectus Phylogeny

P. murrayi occurs in a wide range of Antarctic soil habitats, probably reflecting a high dispersal capacity with high tolerance levels to diverse habitats (Velasco-Castrillón and Stevens, 2014). Despite its abundance and widespread distribution, little sequence divergence was observed within the species and only females were found in most studies (Andrassy, 2012). Based on COI sequence data, *P. murrayi* is highly conserved species that prefers the habitats with moss and higher moisture in Antarctica (Velasco-Castrillón et al., 2014) . With the ability to reside in multiple types of soil habitats in Antarctica, *P. murrayi* has been considered a nematode species with potential to be the dominant nematode species within the MDV under global warming (Nielsen and King, 2015). Furthermore, the population of *P. murrayi* is also sensitive to temperature and soil moisture (Velasco-Castrillón et al., 2014), all aboved demonstrating that it is a better model organism to research evolutionary biology relative to other Antarctic nematodes.

Regarding the overall nematode diversity, only a limited number of genera are known to exist in Antarctica, including *Eudorylaimus, Plectus,* and *Scottnema*. Accordingly, 32 species have been formally recorded from maritime Antarctica and 23 species from continental Antarctica (Powers et al., 1998). The family Plectidae used to be considered a member of the Adenophorea (Dorris et al., 2002), but some studies show that it is more closely related to species belonging to Secernentea (Blaxter et al., 1998). By reconstructing of the phylogenetic tree based on the genome of nematodes, it suggests that *P. murrayi* might be a surviving species close to the beginning of some Secernentean radiation (Phylogenetic tree constructed by Koutsovoulos, 2015, Figure 1). Accordingly, the genus *Plectus* is of particular interest because of its phylogenetic position relative to the origin of the Secernentean radiation (Blaxter et al., 1998; Holterman et al., 2006). *Plectus sp.* (Plectidae) is the sister group to the subsequent lineages of the Secernentean radiation, which produced *Caenorhabditis elegans* and virtually all of the major plant and animal parasitic clades, many of which are scientifically and economically important model species for molecular, developmental and genetic studies (Adhikari et al., 2009b, 2010b; Coolon et al., 2009; Gao et al., 2008; Kim et al., 2014).

Reconstructing the phylogenic tree by the genome of nematodes could provide more evidence to help to resolve the phylogenetic relationship among different species among nematoda. By comparing the genome of *P. murrayi* to other nematode species out of Antarctica, it is possible to find out the evolutionary pattern of stress tolerance genes and understand the adaptation of organism under more complex ecosystems.

We collected *Plectus* samples from different sites of Antarctic Dry Valleys, including Population LB Greek 811, Population LB Greek 813, Population LHS1, Population LHS2, Population battleship 618, Population Taylor Valley, Population Moss, Population Algea,

Population Brownsworth, Population LB Greek 815, Population Onyx. some *P. antarcticus* nematode samples which named on 52A1, 52A2, 52A3, 52A4, 52A5, 52A6, (52B1, 55B2 for *Plectus belgicae*) also analyzed base on 18S, 28S and ITS genes. All our *Plectus* nematodes collected from Dry valleys in Antarctica, including Victoria Valley (Lake Vida); Taylor Valley (Hawes Lake Hoare, Breana Greek, Huey Greek, Onyx river); Wright Valley (Lake Vanda, Lake Brownworth). Nematode samples (table 2) were isolated on site using a modified sugar centrifugation technique and their genus identified using light microscopy and an accepted morphological key. Lysis of the nematodes was performed using 40µl of a proteinase K worm lysis buffer added directly to the same tube and incubated at 56°C until complete digestion of the nematode was verified using light microscopy. Proteinase K was then heat killed by incubating samples at 95°C for 15 minutes. Extracted DNA was stored at -20°C when used regularly and stored at -80°C for long-term periods (> 6 months).

PCR products were analyzed on a 1% agarose gel combined with 4µl of a 10mg/ml ethidium bromide stock added to the warm gel before pouring. Following gel confirmation, 5µl PCR product was cleaned using 2µl of ExoSAP-IT (Affymetrix) according to the manufacturers specifications, and a cycle sequencing reaction was run before samples were submitted to the BYU sequencing center for direct sequencing using big dye v3.1 on a 3730xl genetic analyzer (Applied Biosystems).

36 morphological characters defined in our lab, the matrix shows in supplementary 1. Sequence contigs were assembled using Sequencher v4.10.1 (Gene Codes), aligned using MUSCLE and MAFFT. MUSCLE: iteration 1 and 2 with Neighbor Joining and other with UPGMA. We use default setting for gap penalties in MUSCLE; MAFFT alignment for 18S and 28S by E-INS-i and use G-INS-i for ITS. jModelTest v2.1.4 was used to determine the most

suitable model for each sequence dataset. We chose The Maximum likelihood tree reconstructed using MEGA v5.2. with TrN+G for objective 1 and GTR+G for objective 2 and choose Maximum Parsimony tree as initial tree. In Mrbayes, JC+I+G model for 18S and HKY+G model for 28S and ITS. The parsimony phylogenies were produced using TNT, bootstrap values under replicating resample 1000 times. Trees were edited by Figtree v1.4. The phylogenies in this paper were produced using MrBayes v3.1 (Huelsenbeck, et al.).

The species diversity of *Plectus* genus in Antarctica is still under debate, particularly regards to the validity of the species *P. antarcticus*. Moreover, the distribution of Antarctic *Plectus* nematodes is not completely recorded. We collected nematodes over the course of several years from regions of the McMurdo Dry Valleys in Antarctica. The phylogenetic tree of *Plectus* nematodes from Antarctica and other *Plectus* species was showed in figure 2, it based on 18S, 28S, and ITS genes. Preliminary phylogeny analysis indicates that the species *P. antarcticus* may actually consists of at least two species of *Plectus*, and that these two species are distributed further than originally described. Comparing with other nematodes, *Plectus* nematodes prefer relative higher moisture and lower salinity soil ecosystem, which might consistent with *Plectus* distribution in other regions.

Laboratory Culturing

Previous studies indicated that *P. murrayi* laid eggs 41 to 43 days after hatching, resulting in a total egg-to-egg life cycle length of 53 to 57 days (Yeates et al., 2009). However, we know little about the developmental period of the life cycle of *P. murrayi* in the MDV. The only report of life cycle duration of *P. murrayi* in Antarctica is based on an analysis of populations from Cape Hallet (Yeates et al., 2009). Previous studies show that body length distributions as a

growth time are indicative of an annual life cycle (Adhikari et al., 2010a; Kagoshima et al., 2012).

We used sugar centrifugation method usually to extract *P. murrayi* from soil and sediments (Freckman and Virginia, 1993). After obtaining all nematodes from soil and sediment samples, *P. murrayi* could be picked individually by an eyelash and transferred to sand agar plates (Adhikari et al., 2010a). To maintaining *P. murrayi* on an artificial media (Andrassy, 1998) , the new plates should be put *E. coli* OP-50 on and placed at 37 ˚C for 2 days and then be placed at 15 for ˚C for continue culturing for 3 weeks, making sure to transfer the *P. murrayi* to fresh agar plates every 3-4 weeks to keep the population healthy (Adhikari et al., 2010a; de Tomasel et al., 2013).

Sand on top of the agar plates is required when culturing *Plectus* on a plate in the lab, the reason for which is still unclear. With this method, the *P. murrayi* culturing appears to achieve higher success rates of egg production. Because it is usually difficult to culture Antarctic nematodes, this method could be a reference for growing other bacterivorous nematodes from Antarctica. It takes approximately 5-8 weeks for *P. murrayi* to complete one life cycle on sand agar plates (de Tomasel et al., 2013).

A culture-based strategy could validate recent research that establishes *P. murrayi* as an important model organism for studying adaptation to extreme environmental stress (Adhikari et al., 2009a; Adhikari et al., 2010b). Under this nutrient and water limited soil system, nematodes play an essential role in nutrient cycling, which in turn has a cascading effect on the structure and functioning of its ecological community.

Dessiccation and freezing survival

Besides *P. murrayi*, there are numbers of nematodes that can survive under the low water availability. Because of survival in Antarctic extreme harsh soil habitat, *P. murrayi* also shows high desiccation tolerance among nematodes and shows characteristic features of anhydrobiotic nematodes (e.g., cuticle, body shape) (Treonis et al., 2000; Wharton et al., 2005; Wharton and Raymond, 2015). Given the prevalence of soil nematodes with high cuticular permeability in the Dry Valleys, similar physiological attributes may be widespread among nematodes when assessed under appropriate humidity conditions.

Some studies have reported on the molecular basis of anhydrobiotic survival by investigating the genetic response of *P. murray*i (Adhikari et al., 2009b; Hendriksen, 1983; Sandhove et al., 2016). By sequencing its genome and transcriptomes, it provides insight into the regulation of desiccation-induced transcripts during different stages of stress survival under conditions characteristic of the MDV. Adhikari and his colleagues (2010b) revealed that exposure to slow desiccation and freezing plays a vital role in the regulation of these genes related to signal transduction and help *P. murrayi* survive under freezing and desiccation condition compared to the nematodes suffered fast desiccation and freezing. They also showed that exposure to slow dehydration not only improves extreme desiccation survival but also promotes enhanced cold tolerance (Adhikari et al., 2010b; Wharton and Raymond, 2015).

The transcriptome of *P. murrayi* has been sequenced to identify genes that are differentially expressed during entry into anhydrobiosis. *P. murrayi* showed differential expression of a suite of genes and continuous up-regulation of others upon exposure to desiccation and freezing, which consistant with the results of previous studies (Adhikari et al., 2009b, 2010b). In Adhikari's study, there are some genes in *P. murrayi* related to desiccation

stress up-regulated during dehydration, such as the genes encoding trehalose-6-phosphate synthase, aldehyde dehydrogenase, glycerol kinase, malate synthase, heat shock proteins and a novel protein, and a gene encoding an antifreeze protein was down-regulated (Adhikari et al., 2010b).

To understanding the cold tolerance of frozen *P. murrayi*, Wharton and Raymond (2015) found that they contained on average smaller ice spaces than *Plectus redivivus* and starved *Panagrolaimus sp* and have higher survival rate. Survival of intracellular freezing could be more common among cells and tissues which were frozen for cryopreservation than is currently realized, as the formation of small ice crystals is not observed using optical cryomicroscopy (Salinas-Flores et al., 2008). Kagoshima et al. (2012) also showed that *P. murrayi* could survive exposure in the frozen state to the biologically unrealistic temperature of -80°C, although at a relatively low rate (19.2 %). The free-living fungivorous nematode *Aphelenchus avenae* has been shown to accumulate large amounts of the disaccharide trehalose, a late embryogenesis abundant (LEA) protein and a novel protein named anhydrin when exposed to a moderate reduction in RH (Goyal, 2005). The Antarctic nematode *Panagrolaimus davidi* which, like *P. murrayi*, can survive both desiccation and freezing, expresses an ice-active protein that shows recrystallization inhibition during freezing (Wharton et al., 2005).

We know*P. murrayi* faces to one of the most extreme and unpredictable terrestrial environments on earth (Priscu, 1998) and to adapt to such extreme conditions it might have evolved a mechanism to maintain some stress responsive genes with lower metabolism and growth rate (Buckley et al., 2004), which happens in ciliates (La Terza et al., 2001), yeast *Candida psychrophila*(Deegenaars and Watson, 1997) and the Antarctic midge *Belgica antarctica*(Rinehart et al., 2006). It could be a common adaptive strategy of Antarcitc animals.

The capacity to survive prolonged periods of low moisture availability and freezing temperatures is of considerable adaptive significance to *P. murrayi*. The survival of nematodes in these conditions encompasses differential expression of a suite of genes from different functional groups and constitutive expression of others. Their adaptations to desiccation stress can promote enhanced freeze survival, and provide evidence that slow dehydration enhances the lower limit of freeze tolerance in an Antarctic nematode. Furthermore, such conditions can influence the survival of significant water loss, and permit the identification of subtle desiccation and cold tolerance strategies employed by Antarctic biota.

Prospectus

Most of the Antarctic Dry Valley nematodes are known for their ability to survive desiccation and freezing conditions in an anhydrobiotic stage(Treonis and Wall, 2005). Mechanisms that control the entry into, maintenance and recovery from anhydrobiosis are poorly understood. *P. murrayi* lives in one of the most extreme environments on Earth, where it faces a variety of stresses including freezing, desiccation, osmotic shock, ultraviolet radiation, continuous light or dark, low nutrient availability, unstable substrate, etc. It is not clear for how long *P. murrayi* might tolerate freezing. Samples of the Antarctic moss, *Bryum argenteum*, were collected on 1 October 1983 at Langhovde, Soya coast and eastern Antarctica were stored at -20°C. After 25.5 years of storage, living nematodes were recovered from the samples and were identified as *P. murrayi* by morphological examination and nucleotide sequencing of ribosomal RNA loci.

The fact that *P. murrayi* occurs in a wide range of soil habitats probably reflects a high dispersal capacity and broad ecological amplitude (Velasco-Castrillón et al., 2014). However, little is known of its developmental biology and evolutionary adaptation at the genomic level.

Genomic and proteomic approaches could provide valuable information, such as functional genes characterization and genome decay under extreme cold and dry environment. With the development of sequencing technology and bioinformatics tools, sequencing genome and transcriptome of *P. murrayi* is more operatable, and more genes involved in extreme environmental stress will be identified.

We have a strong understanding of the phylogenetic position of *Plectus*, its distribution, habitat niche breadth, and methods to culture them in the lab. Next-generation sequencing allows for improved genome sequencing and assembly, even from samples with low concentration. When considered together, these developments could facilitate dramatic improvements in understanding gene-level responses to extreme environmental stressors.

Under elemental nutrient limitation in Antarctica, we know that certain genotypes or species perform better under high phosphorus (P) conditions (Jeyasingh and Weider, 2007), but we know little about the standing genetic variation in natural populations for developmental performance under contrasting P regimes. Therefore, more sophisticated work is needed to find the specific mechanisms connecting genomic divergence and organismal development. Also, revealing the mechanism of their stress tolerance strategies and participating in nutrients cycling at genomic level is important for researchers to understand their evolutionary messages, such as the evolution pattern of genes related to stress tolerance and understanding growth rate hypothesis at genomic level. Studying *P. murrayi*, a unique Antarctic nematode, will allow us to understand the role of stoichiometric relations and nutrient cycles in shaping organismal development, and the organization of trophic interactions and, ultimately, ecosystem structure and functioning.

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Figures and Tables

Figures

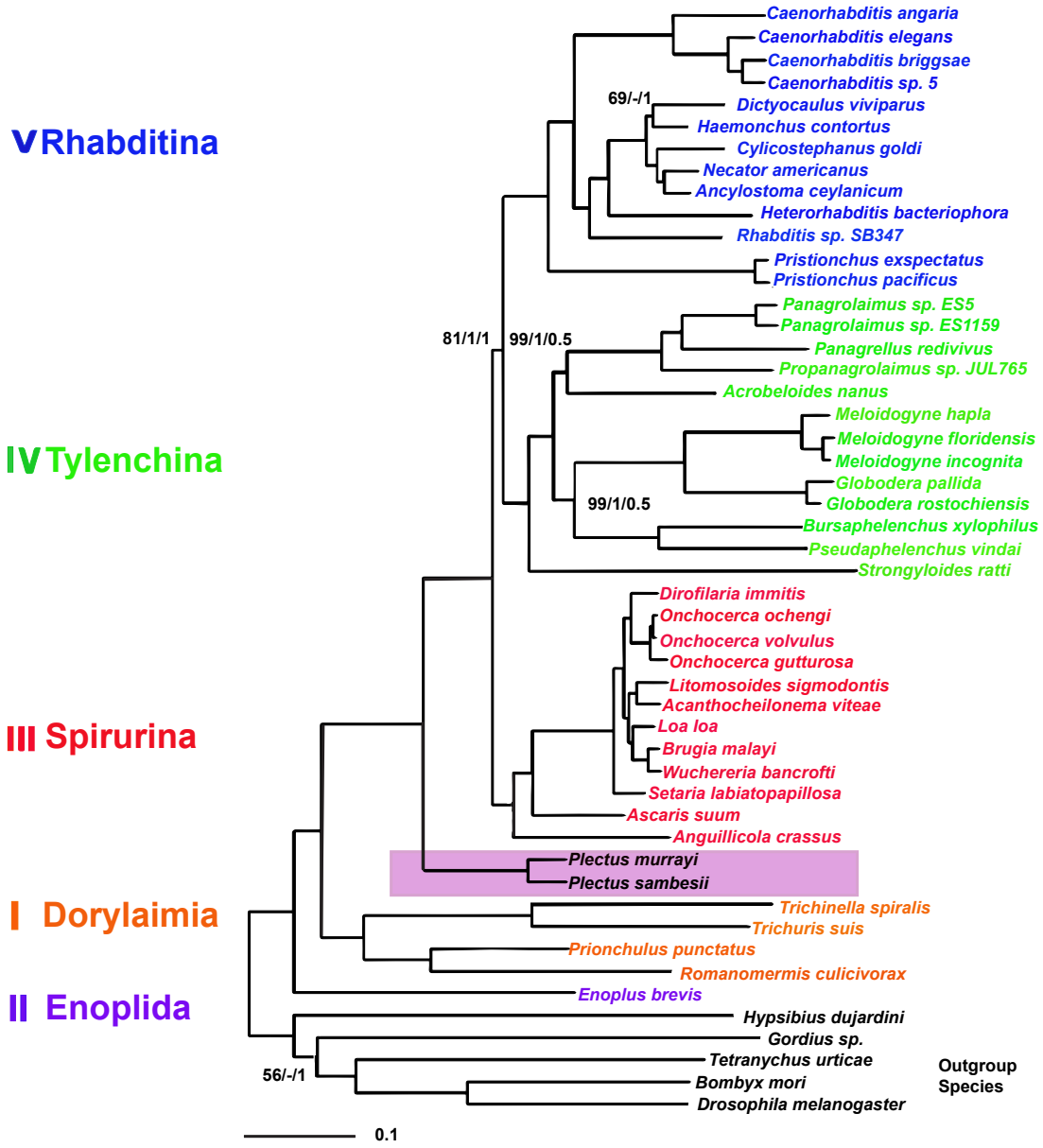

Figure 1. Nematode phylogeny modified from Koutsovoulos' work (Koutsovoulos 2015), RAxML bootstraps values below 100 or PhyloBayes posterior probabilities below 1 are shown on the nodes.

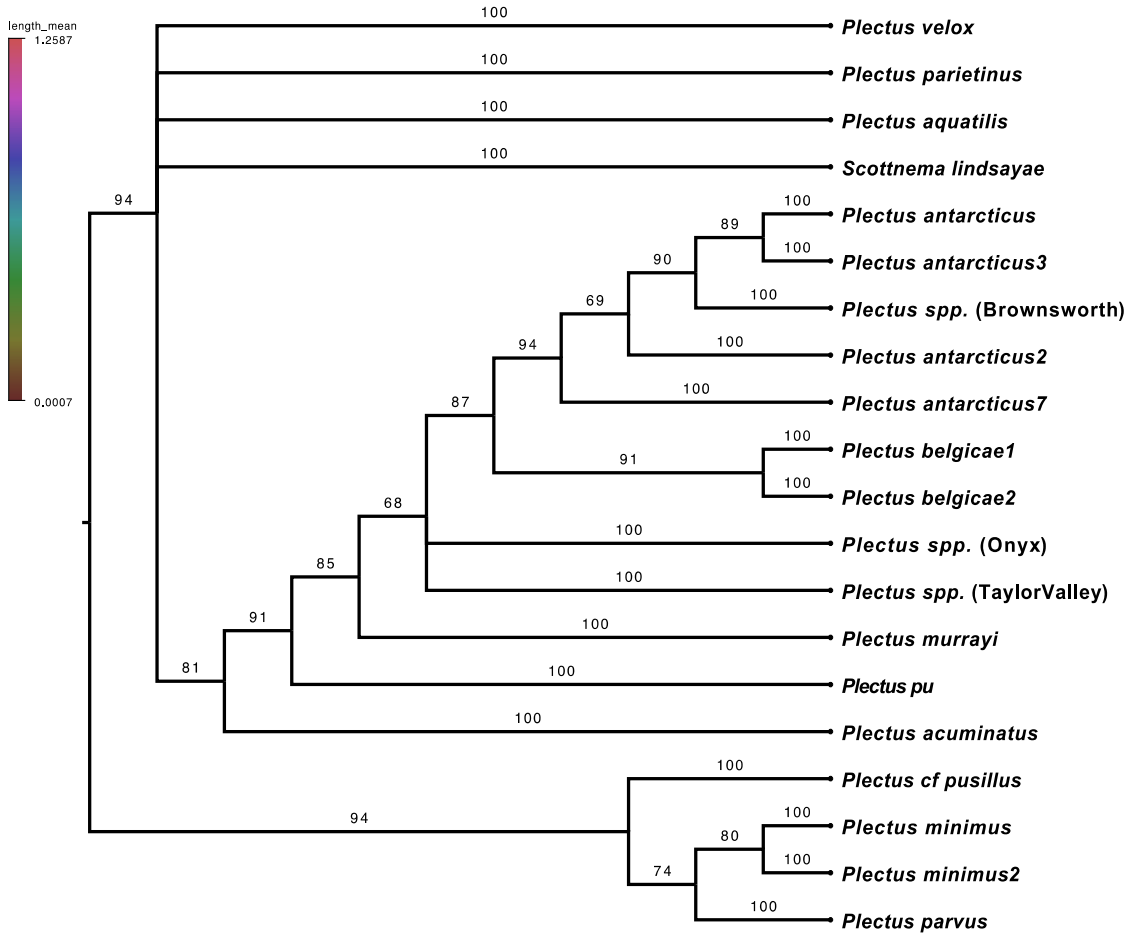

Figure 2. Bayesian tree based on combined analysis of 18S, 28S and ITS for *Plectus*. Posterior probabilities are shown at some nodes.

Figure 3. *Plectus murrayi*, a nematode from Antarctic MDV

Tables

Table 1. Biogeographic distribution of *Plectus murrayi* in Victoria Land, Antarctica. (Cited and modified from Adams et al., 2014 with consent (Adams et al. 2014))

An abundance is per kilogram of soil, Low = >0 to 20 nematodes per kg dry soil, M-low = 21 to 200 nematodes per kg dry soil, Media = 201 to 600 nematodes per kg dry soil, M-high = 601 to 1000 nematodes per kg dry soil and high = 1001 to 2000 nematodes per kg dry soil.

Biogeographic Location	Habitat	abundance	reference
McMurdo Dry Valleys			
Alanta Valley	Soil	present	Freckman and Virginia, 1993
Battleship Promontory	Soil	Low	Adams, et al., 2014 collected in 1993
East, middle and southwestern end	Soil	Low	Adams, et al., 2014 collected in 1993
Garwood Valley			
Garwood Lake	Soil	M-low	Adams, et al., 2014 collected in 1993
	Soil	M-low	Wall Freckman and Virginia, 1998
	Soil	M-low	Adams, et al., 2014 collected in 1993
Miers Valley			
Miers Clacier	Soil	M-low	Adams, et al., 2014 collected in 1993
Taylor Valley			
Canada glacier			
Near the glacier	Windblown sediment on top of glacier	Present	Adams, et al., 2014 collected in 1993
Waterfall (upper west)	Cryconite hole	Present	Adams, et al., 2014 collected in 1993
Lake Bonney	Soil polygon crack	Low	Adams, et al., 2014 collected in 1993
	Soil	Media	Adams, et al., 2014 collected in 1993
West lobe	Soil	Low	Adams, et al., 2014 collected in 2002
	Soil and stream sediment	M-low	Adams, et al., 2014 collected in 2000
	Soil	Low	Adams, et al., 2014 collected in 2003
	Soil and sediment	M-low	Adams, et al., 2014 collected in 2002
Lake Chad	Soil	M-low	Adams, et al., 2014 collected in 1995
Lake Fryxell	Soil	M-high	Adams, et al., 2014 collected in 1990

	Soil	Low	Adams, et al., 2014 collected in 1993
	Soil	Media	Adams, et al., 2014 collected in 1995
Huey Creek	Soil	M-low	Adams, et al., 2014 collected in 1999
Harnish Creek	Soil and sediment	M-low	Adams, et al., 2014 collected in 2000
South side	Soil	Low	Adams, et al., 2014 collected in 2000 and 2002
South side	Soil and sediment	High	Adams, et al., 2014 collected in 2002
South side	Soil	Low	Adams, et al., 2014 collected in 2003
South side	Soil	Low	Adams, et al., 2014 collected in 2003
South side near Green Creek	Soil	Media	Adams, et al., 2014 collected in 2003
Green Creek	Soil	Media	(Barrett et al, 2006c)
Lake Hoare			
North side	Soil	low	Adams, et al., 2014 collected in 1993
South side	Soil	Low	Powers et al. 1995a
South side	Soil at varying elevation	Low	Adams, et al., 2014 collected in 1995, 1998 and 2002
North side	Soil	low	Courtright et al. 1996
South side	Soil	Low	Powers et al. 1998
North side	Soil	M-low	Adams, et al., 2014 collected in 1999
North side	Soil	Low	Courtright et al. 2001
South side	Soil	Low	Adams, et al., 2014 collected in 2001
South side	Soil	Low	Porazinska et al. 2002b
	Soil	high	Adams, et al., 2014 collected in 2002
South side	Soil	low	Adams, et al., 2014 collected in 2003
	Soil	M-low	Wall Freckman and Virginia 1998
	Soil	M-low	Treonis et al. 2000
	0-5 cm soil subnivian	M-low	Gooseff et al. 2003
Taylor Glacier	Windblown sediment on top of glacier	present	Adams, et al., 2014 collected in 1998
Victoria Valley	Soil	Present	Adams, et al., 2014 collected in 2003
Wright Valley			
Bull pass	Soil	M-low	Adams, et al., 2014 collected in 1990

Lake Bull	Soil	low	Adams, et al., 2014 collected in 2003
	Soil	M-low	Wall Freckman and Virginia 1998
Koettlitz Glacier and Southern Coastal regions			
	Moss (Bryum antarcticum)	high	Yeates 1970
	Mossy soil and melt pools with abundant algae (Nostoc commune)	present	Timm 1971
Northern Coastal Region			
Cape Hallett			
	Soil	Media	Barrett et al. 2006c
	Soil amongst penguin rookery	M-low	Adams, et al., 2014 collected in 2003
Edmonson Point			
	Wet moss near a brook	present	Vinciguerra 1994
	Soil	M-high	Adams, et al., 2014 collected in 1996
	Soil	M-low	Adams, et al., 2014 collected in 2001
Luther Peak	Soil	Low	Adams, et al., 2014 collected in 2003
Luther Vale South	Soil	Low	Barrett et al. 2006c
Shackleton glacier			
Heekin Valley	Soil	Present	Collected in 2017
Mt. Franke	Soil	Present	Collected in 2017
Mt. speed	Soil	Present	Collected in 2017
Mt. Wasko	Soil	Present	Collected in 2017
Environmental History			
Canada Glacier	Soil	Present	Collected in 2017
Asgard Helo Pad	Soil	Low	Collected in 2017
Meserve Glacier Helo Pad	Soil	Media	Collected in 2017
Meserve Glacier Camp	Soil	Low	Collected in 2017

Table 2. Different populations for *Plectus* samples in Antarctica

Plectus population	Location	Sequences (18S, 28S, ITS)	Number of Sequence
Population Onyx	Taylor Valley	Lab generated	6
Population Breana_Creek	Taylor Valley	Lab generated	10
Population Taylor_Valley	Taylor Valley	Lab generated	2
Population Brownsworth	Wright Valley	Lab generated	5
Population Battleship	No record	Lab generated	8
Population Algae	No record	Lab generated	15
Population Lake Hoare	Taylor Valley	Lab generated	4
Population Moss	No record	Lab generated	5

Table 3. *Plectus* species analyzed in this paper

Plectus species	18S	28S	ITS
Plectus murrayi	2	1	4
Plectus Antarcticus	5	3	6
Plectus minimus	KC206040.1	1	No reported
Plectus Pu	4	4	1
Plactus aquatilis	2	1	No reported
Plectus velox	JX678608.1	No reported	No reported
Plectus Parietinus	AY146551.2	No reported	No reported
Plectus Parvus	AY919236.1	No reported	No reported
Plectus acuminatus	AB477087.1	AB477070.1	No reported
Plectus cf Pusillus	AY284705.1	No reported	No reported
Plectus Hm	4	4	2
Scottnema lindsayae	HQ270133	HM439773	No reported

Chapter 2

Elemental Stoichiometry as a Driver of Life History Evolution: An Experimental Test of the Growth Rate Hypothesis

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Abstract

Elemental stoichiometry provides a useful framework for understanding sources and controls of nutrient availability. The prior *in situ* research on populations of the Antarctic soil nematode *Plectus murrayi* revealed a link between cellular phosphorus (P) and organismal development as postulated by the growth rate hypothesis (GRH). We predict that in a P-deficient environment we will find lower cellular RNA concentrations and that natural selection will have reduced RNA gene copy number in the genome, and consequently lowered rates of overall gene expression. To test the GRH in *P. murrayi* and *Caenorhabditis elegans* under laboratory conditions, we manipulated the amount of available P to simulate that found in the Dry Valleys, and to see if we could identify some of the specific mechanisms connecting elemental stoichiometric constraints

and nematode ontogeny. We found that the number of copies of the 18S ribosomal DNA tandem array in *C. elegans* cultured in a P-poor environment was over 12 times less than in populations reared in a P-enriched environment. *P. murrayi* also evolved a decrease in 18S ribosomal DNA copy number, although not as dramatically as *C. elegans*. Additionally, the life span and body size of *C. elegans* and *P. murrayi* reared in excess P were significantly different from those reared in P-poor conditions. Our findings underscore the critical relationship between the evolution of life history traits and the GRH as well as the role of elemental stoichiometry in shaping the organization of trophic interactions, ecosystem structure and functioning.

Introduction

All organisms are composed of elements. As the grow, their somatic elemental ratios reflect underlying biochemical allocations that are produced to meet the demands of development. Decades of research on a wide range of organisms and communities have shown the importance of elemental composition for explaining and predicting ecological patterns and processes (Waite and Sack, 2010). Stoichiometric approaches are based on the general premise that organisms influence and are impacted by the available nutrients from the environment, primarily such as carbon, nitrogen and phosphorous. Additionally, the cellular and biochemical machinery required for divergent life history strategies drive the stoichiometric requirements of individual organisms (Schindler, 2003). Each cell regulates ribosome and protein synthesis to maintain a balance of nutrition and development, which plays an essential role in the growth rate and development of every organism. However, the mechanisms underlying/behind the relationship between growth rate and ribosome synthesis under different stoichiometric limitations are poorly understood (Mathis et al., 2017). Some studies showed there is a connection between organism from protozoa to mammals longevity and development and protein synthesis rates (Dai et al., 2014; Edwards et al., 2015; Karunadharma et al., 2015; Price et al., 2012; Selman et al., 2009). Moreover, nutrient signals could impact ribosome maintenance (Mathis et al., 2017) by manipulating the cellular processes of ribosome building. Elevated demands for increased allocation to Phosphorus (P)-rich ribosomal RNA under rapid growth drives variation in the P content of many biotas. Stoichiometry and growth rate variation are related aspects of the ecology and evolution of consumer-resource interactions. Ecological stoichiometry refers to the relationship between multiple chemical elements and growth rate in all organisms (Elser et al., 2000). Several studies have shown that the C : N : P ratios of primary

producers vary markedly in terrestrial, marine, and freshwater ecosystems (Zimmerman et al., 2013), whereas herbivores show much less variation in elemental composition (Elser et al., 2000; Sterner and George, 2000; Sterner and Robinson, 1994). According to the dominant paradigm, P is the primary limiting element in freshwater systems and post-glacial landscapes (Schmidt et al., 2016), phosphorus availability also deals with the denitrification and associated N_2O emissions in soil systems (Mehnaz and Dijkstra, 2016), and C : P ratios suggestive of P limitation of herbivores (Elser et al., 2009; Schindler, 2003; Sterner, 1997; Sterner and Hessen, 1994) are commonly found in lakes (Elser et al., 2000; Poxleitner et al., 2016).

The growth rate hypothesis (GRH), which states that available P content in the environment is directly coupled to high demands for P-rich ribosomal RNA in fast-growing organisms, has been suggested as an adaptive explanation for variation in the P content in consumer taxa (Elser et al., 2000). According to the GRH, organisms with high P demands should possess the higher growth rates under P-rich resource conditions, but should also be more sensitive to resource P limitation, which shows that high biomass P-content reflects an increased allocation to P-rich ribosomal RNA that is needed to meet the protein synthesis demands of increased development. The prediction is that, across taxa, maximum growth rates should increase with environmental P content (Elser et al., 2000). However, this relationship may disappear or even reverse under P limitation. Although several studies have examined the consequences of dietary P deficiency at moderate to high food levels, few studies have examined P limitation in organisms at scarce nutrients resources. Further, these studies have all focused on Daphnia or plants (Tao et al., 2016), which has also restricted testing of the GRH. Besides studying on the relationship between fecundity and life span (Boonekamp et al., 2015; Flatt, 2011; Labbadia and Morimoto, 2015), there are some studies on the life history traits, such as the

growth rate of development and the relationship between body size and life span (Lee et al., 2013; Ricklefs, 2006). Furthermore, Lind and his colleagues (Lind et al., 2017) found that unlike fecundity, the growth rate of *Caenorhabditis remanei* was slower when their life span was longer under heat-shock stress, which suggests that the growth rate could be an important life history trait under environmental stresses.

Earlier studies have found that gene copy number varies commonly in almost all eukaryotic organisms, such as human (Schrider and Hahn, 2010), mouse (Graubert et al., 2007), maize (Springer et al., 2009), yeast (Carreto et al., 2008). The association between variation of ribosomal RNA gene copy numbers and its potential consequences have also been studied previously (Coenye and Vandamme, 2003; Pei et al., 2010). Since ribosomal RNA genes are widely used for the study of evolutionary history and taxonomic assignment of individual organisms, it is interesting to consider the implications of this rRNA gene copy number variants on phylogenetic studies along with GRH. Tourova (2003) confirmed that ribosomal copy numbers increased during accelerated growth rate in organisms. Furthermore, in *E. coli* it is known that multiple rRNA operons could express sufficient ribosomes and achieve higher growth rates (Schirrmeister et al., 2012).

Nematodes are one of the most widely distributed organisms around the world: they have been reported from the almost every continent (Bongers and Ferris, 1999). Their simple structure and transparent bodies make them excellent model organisms for studying developmental and embryonic cell lineage evolution. The McMurdo Dry Valleys (MDV) is one of the most extreme terrestrial environments on earth. Here, physical conditions confer significant environmental stress on biota. Because of their poor capability to support life, these valleys are thought to have the lowest plant and invertebrate species diversity on earth (Adams et al., 2006; Schmoldt et al.,

1975). A unique and extreme environment in the MDV soil habitat exerts strong selection on every organism surviving there: for instance, freezing temperatures, strong stoichiometric constraints, limited moisture availability, salt accumulation and desiccation. It is conceivable that only organisms capable of tolerating all of these stressors have been able to persist in the Antarctic soil ecosystem. All of the above make the MDV soil ecosystem a natural laboratory for testing the role of stoichiometric constraints as drivers of genome architecture and life history traits. Nematodes are also widespread in the MDV and their distribution is regulated by abiotic factors such as soil moisture, elemental stoichiometry, salinity and pH. *Scottnema lindsayae*, the most widely distributed of the MDV nematodes, dominants drier, saltier soil, while *Plectus murrayi* tends to be found in more productive, moist areas (Adams et al., 2006; Barrett et al., 2007; Treonis et al., 1999; Velasco-Castrillón and Stevens, 2014).

Bioavailable phosphorus (P) differs significantly in MDV ecosystem, both in terrestrial and aquatic, it has been found in a wide range of age of this landscape surfaces, and various landscape geochemistry have different nutrient availability, and it is very likely influenced by till composition and age (Barrett et al., 2007; Bate et al., 2008). Some studies showed the amount of P is higher in Fryxell basin than it is in Bonney basin. Fryxell basin soluble P findings correlated positively with the Al-bound phase, possibly facilitating P availability to microfauna. The P fraction distribution in both the Fryxell and Bonney basins matches/reflects the general relationship between weathering intensity and P distribution of other arid ecosystems (Blecker et al., 2006).

P availability imposes strong constraints on the organisms living in the MDV. *P. murrayi* has been found in both the P-rich (Lake Fryxell basin) and P-poor (Lake Bonney basin) areas (Gooseff et al., 2003; Hogg and Wall, 2011; Treonis et al., 1999), the temperature of these two

areas are similar throughout the whole year. Interestingly, populations of *P. murrayi* found under different stoichiometric P conditions exhibit different growth rates and average body size (Freckman and Virginia, 1997). Previous studies documented that *P. murrayi* in the wild have a multiple year life cycle with slow production of eggs in relatively few numbers. Under laboratory conditions, it takes 6-8 weeks at 15˚C to complete a developmental cycle (Adhikari et al., 2010).

Our studies on the GRH underscore the important relationship between the evolution of life history traits and gene copy number, as well as the role of elemental stoichiometry, in shaping the organization of trophic interactions and, ultimately, ecosystem structure and functioning in soil ecosystems. We have already detected intraspecific processes leading to potential shifts in the competitive dominance of different genotypes, resulting in the selection that favors changes in individuals at the genomic level over time that is linked to their developmental rate. These evolutionary developmental processes influence genomic architecture, and subsequently establish a basis of reciprocal feedbacks between their environment and evolutionary change. Prior *in situ* research on natural populations of the Antarctic soil nematode *P. murrayi* revealed a link between cellular P and organismal development as postulated by the GRH (Barrett et al., 2007). In order to test the generality of this hypothesis, we set out to see if we could use an experimental evolution approach to replicate in the laboratory what had been observed in nature. Because even under the best conditions field and laboratory-reared populations of *P. murrayi* are relatively slow growing, we replicated our experiments with the more rapidly growing nematode, *C. elegans*. We tested this hypothesis in the laboratory at the same temperature (15 ˚C) by culturing them on P-rich and P-poor media and periodically measuring life history traits and 18S rRNA expression.

In accordance with the GRH, we hypothesize that in a P-poor environment, both nematodes will grow more slowly but achieve a larger body size at maturity. We also predict that in a P-deficient environment we will find lower cellular RNA concentrations and that selection will reduce the number of copies of RNA genes in the genome, and subsequently lower rates of overall gene expression

Results

Our experimental design includes four populations, two each per nematode, grown up in two levels of P concentration, P-rich and P-poor. PE is for *P. murrayi* in P-rich media, PL is for *P. murrayi* in P-poor media; CE is for *C. elegans* in P-rich media, CL is for *C. elegans* in P-poor media. In addition to culturing nematodes at different P concentrations, we tracked 18S rRNA expression for each group, we ran 16 tests on *C. elegans* (Figure 1) and 10 on *P. murrayi* (Figure 2) by Real-time qPCR with ß-actin as inner reference gene.

We found that the number of copies of the 18S ribosomal DNA tandem array in *C. elegans* reared in a P-poor environment is 13.92 times less than populations reared in a P-rich environment (p-value = 2.2e-16) (Figure 1) (Wilcoxon test, $P<0.0001$). Under similar conditions rDNA gene copy number in *P. murrayi* also decreased, although not as dramatically, to 0.24 times less than populations from a P-rich condition (p-value = 0.065) (Figure 2) (Wilcoxon test, $P>0.05$). Meanwhile, the copy number of inner reference (ß-actin) are almost identical in both *C. elegans* and *P. murrayi*, which shows that there was not significant signal contamination from primers or other PCR regents.

We measured fecundity for these populations and found that those cultured in limited phosphate condition have more extended reproduction periods and slower reproduction rate per day (Figure 3-A, B). Both *C. elegans* and *P. murrayi* reached reproduction peak per day earlier

in P-rich than in P-poor media. The population living in phosphate excess had shorter reproduction period and faster reproductive rate, though not significantly ($P>0.05$). Nevertheless, these two populations had similar amounts of total eggs (Figure 4-A, B) (Paired t-test, $P>0.05$). The average total number of eggs was 122.6, 136.2, 19.33, 19.8 for CE, CL, PE, PL respectively.

Although there is no significant difference in the total number of eggs between nematodes reared in P-rich and P-poor media, we found the nematodes reared in P-poor media produced more slowly than those raised in P-rich media. Also, we observed and record their egg volumes (Figure 5-A, B) and hatch rates (Figure 6-A, B) for each nematode group. The average egg size of *C. elegans* in P-rich and P-poor was 1.6×10^{-5} mm^3 and 0.93×10^{-6} mm^3, and for *P. murrayi* 1.59×10^{-5} mm^3 and 1.08×10^{-5} mm^3 respectively. The average hatch rates for *C. elegans* and *P. murrayi* reared in P-rich and P-poor media were 93.71%, 91.70%, 70.93% and 57.30% respectively.

The adult body volumes of both *C. elegans* and *P. murrayi* reared in P-rich were significantly smaller than those reared in P-poor conditions (t-test, $P<0.05$), while body length displayed the opposite trend (t-test, $P<0.05$) (Figure7-A, B, C, D). Moreover, the nematodes required longer growth times to reach reproductive maturity in P-poor conditions than when grown in P-rich condition.

By tracking the developmental period of these four groups, our results showed that there are significant differences between some life stages of development for both *C. elegans* and *P. murrayi*, but not every stage (Figure 8, 9). In *C. elegans*, the time required to develop from egg to young adult and from egg to death is different. *C. elegans* reared in P-rich media have longer longevity and reach the young adult stage earlier than those grown in P-poor media. In *P.*

murrayi, the time from egg to young adult, to adult and egg laying are different. It took less time for *P. murrayi* growing in P-rich media to reach those stages than those grown in P-poor media.

Discussion

Our experimental evolution results for *C. elegans* are consistent with our understanding of resource availability and the GRH. Stoichiometric constraints have been shown to be correlated with life history traits and organisms cultured in the lab should possess the same traits as those found in wild P-poor environments, i.e., delayed maturity and larger adult body size/volume. It means the phosphate concentration in different habitats could affect the population developmental processes of nematodes. Additionally, the nematodes cultured with more P achieve their peak fecundity earlier than the ones with less P, which might imply that more available P resources to build their proteins, more energy to reproduce, and faster reproduce comes along. Under the same temperature, nematodes cultured in P-rich environment possess a higher volume and shorter while the nematodes cultured in P-poor are thinner and longer. This phenomenon could be an adaptive strategy of development that nematodes utilize to respond to environmental stoichiometric constraints by reallocating resources and energy in such a way that they can still develop. Both *C. elegans* and *P. murrayi* could response to environmental P constraints by changing life history traits and the strategies to adapt to resources limitation, which are consistent with GRH found in Antarctic Dry Valleys.

The nematodes live in P-poor have longer reproduction period than the ones live in P-rich condition, while the total number of eggs produced by those groups is not significantly different. Accordingly, we suggest that for both *C. elegans* and *P. murrayi*, P resources could impact their reproduction by slowing their development when there is not enough P. Previous studies show that changes in growing temperature could lead to altered life history traits on nematodes, and

under different environmental stresses the growth rate and development could respond (Ball and Baker, 1996; Lind et al., 2017; Lind and Johansson, 2011) . Moreover, nutrient availability can drive microbial community structure and composition, which is important when considering development and reproduction in nematodes(Shapira, 2017). In our experiment, the temperature was 15 ˚C for culturing and experiments, which reflects the ambient soil temperature of the MCM dry valleys for the two locations with different P content is similar. Some researchers consider that life span will decrease when the organisms invest more resources into reproduction (Boonekamp et al., 2015; Flatt, 2011), however, finding an experimental fact to support this point is not easy (Flatt, 2011; Maklakov and Immler, 2016). Development and duration of juvenile growth are responsible for the differences in life span. Lind and his colleagues (Lind et al., 2017) suggested that development showed a strong response to stress selection and had a significant effect on longevity. In our study, the life span of *C. elegans* differs between P-poor and P-rich conditions, while the life span of *P. murrayi* in different P conditions is similar. Moreover, since the growth rate of both *C. elelgans* and *P. murrayi* in P-poor is slower than in P-excess, we suggest that P availability plays an essential role in their development and reproduction, and since *P. murrayi* is endemic to Antarctica, it might have evolved a unique strategy to adapt to this incredibly harsh habitat.

There was a significant increase in 18S expression level in *C. elegans* grown in P-rich media compared to those under P-poor conditions, which suggests that GRH could be a typical pattern among nematodes. According to GRH, we predicted that there would be higher 18S gene expression in nematodes cultured under P-rich condition, however, a lack of change in expression in *P. murrayi* (Figure 2) is not as supportive of this interpretation (Figure 1).

In our experiment, the ΔCt value of 18S rRNA copy number in CE is significantly higher than the ΔCt value in CL while the ΔCt value of 18S ribosome in PE is not that significantly different from the ΔCt value in PL as *C. elegans*. However, changes in certain life history traits in *P. murrayi* correlate to differences in stoichiometric P constraints, which demonstrates that *P. murrayi* has a higher growth rate in P-rich conditions than those grown with limited phosphorus. Since *P. murrayi* has a relatively longer generation time (6-8 weeks under 15˚C) than *C. elegans* (72-96 hours under 15˚C), it could take longer for *P. murrayi* to respond to changes in P availability. It is highly possible that an increase in 18S expression in *P. murrayi* could be detected after additional generations. Additionally, by detecting 18S rRNA expression level we tried to see how the ribosome building in nematodes growing in different P condition, besides the genetic coding factors, the stoichiometric P constrains impact on the development of nematodes, which may also interrupt epigenetic programming in nematodes. That also could be a reason for the non-significant difference in18S rRNA expression between *P. murrayi* in P-poor and P-excess.

Understanding how organisms respond to stoichiometric constraints in both the short and long-term could shed light on ecological drivers of evolutionary development. We hope to be able to explore new connections between functional genes that are responsible for differential development in different habitats, and further explore stoichiometric constraints on development (including the GRH and genome evolution). Furthermore, we also tried to test our hypotheses along with the genomic data and currently have the whole genome of *P. murrayi* sequenced and annotated. The experiments undertaken include a transcriptomic analysis of the *P. murrayi* cultured in different temperature and P situations.

This research will underscore the important relationship between the evolution of life history traits and genome organization, as well as the role of elemental stoichiometry in shaping the organization of trophic interactions and, ultimately, ecosystem structure and functioning.

Materials and Methods

C. elegans and *P. murray* is Culturing:

We maintained the *C. elegans* strain N4 and *P. murrayi* for different environmental phosphorous(P) experiments in two P concentration media for at least 30 months. It takes 6-8 weeks for *P. murrayi* to finish a generation time, while *C. elegans* requires only 4 days. We inoculated prepared excess/limited phosphorous agar plates, added 30µL of stock OP-50 to phosphorous plates, used a cotton swab to spread the bacteria on the plate and incubated them at 37˚C for 2 days. Then we transferred nematodes onto the plate and incubated them at 15˚C for 3 weeks before repeating the process for the next (Adhikari et al., 2010).

We used phosphorous sand agar media for growing nematodes, which includes 15g Agar, 965 mL H_2O, 20 ml BMB for both P-poor and P-rich plates, and 1.033 mg K_2HPO_4 for P-poor and 10.33 mg K_2HPO_4 for P-excess. The pH was adjusted 7.0, H_2O was added to 1.0 L, and the mixture was autoclaved for 20 minutes at 120 ˚C. Sand was poured on cooled plates, which were then stored in 4 ˚C.

RNA extraction and real-time polymerase chain reaction:

Nematodes stored in RNAlater solution were washed twice with 5% solution of phosphate buffer saline (PBS) before RNA extraction. Total RNA for Quantitative real-time PCR was extracted using Trizol reagent (Molecular Research Center Inc.). Reverse transcription (RT) was performed with 1ng of total RNA from each specimen. RT reaction of polyadenylated

mRNA to cDNA was done using the ImPromp-IItm reverse transcriptase and random hexamer primer.

qRT-PCR was performed with LightCycler 480 SYBER Green 1 Mastermix (three replicate samples for each extraction) and gene specific primers in a light cycler 480 RT-PCR system (Roche Applied Science) equipped with LightCycler 480 software with the following program: 3 min at 95˚C; 45 cycles of the 30s at 94˚C, 30s at 55˚C and 1 min at 72˚C followed by a standard melt curve. To minimize mRNA quantification errors, genomic DNA contamination biases, and to correct for inter-sample variation, we used nematode ß-actin genes as an internal control. We collected all Ct values from qPCR and duplicated each group more than 12 times. The distribution of the Ct data is not strictly normal, we applied the Wilcoxon test to normalize. We extracted threshold cycle (Ct) values for each group of samples, and ran a t-test and Wilcox test on them in R (RStudio Team, 2015). The ΔCt values of 18S ribosome of *C. elegans* were tested by Fisher exact test, and the ΔCt values of the copy number of 18S ribosome of *P. murrayi* were tested by Chi-square test.

Life history traits observation:

We collected *P. murrayi* from the MDV during 2008-2009. The samples were taken by clean plastic scoops from the soil up to 10-cm depth, and placed in sterile 24-oz Whirlpack bags, sealed and shipped frozen at -20˚C to Brigham Young University. Four groups of nematodes (two groups of *C. elegans*, two groups of *P. murrayi*) were grown at 15°C, which has been found to be the optimal growing temperature for *P. murrayi* (Adhikari et al., 2010; de Tomasel et al., 2009). One group of nematodes were grown in P-poor plates and the other group are in P-rich plates. In each group, pregnant females were picked into fresh petri dishes with media seeded with OP-50 *Escherichia coli* as a food source. All plates were checked daily to track when the

eggs were laid. After juveniles hatched, they were transferred by picking into new plates. Afterward, photos were taken daily and their body sizes were measured using a CKX 41 Olympus inverted microscope.

The duration time of each stages and the productivity of each of the four groups were observed and recorded; their body sizes - including body lengths and volumes - were measured and calculated by the modified Andrássy's formula (de Tomasel et al., 2013) in MatLab (Yakimenko, 2011). The differences between each group were analyzed by t-test ($P < 0.05$) in R (Horton and Kleinman, 2015). And Hochberg correction was applied on each test.

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Figures

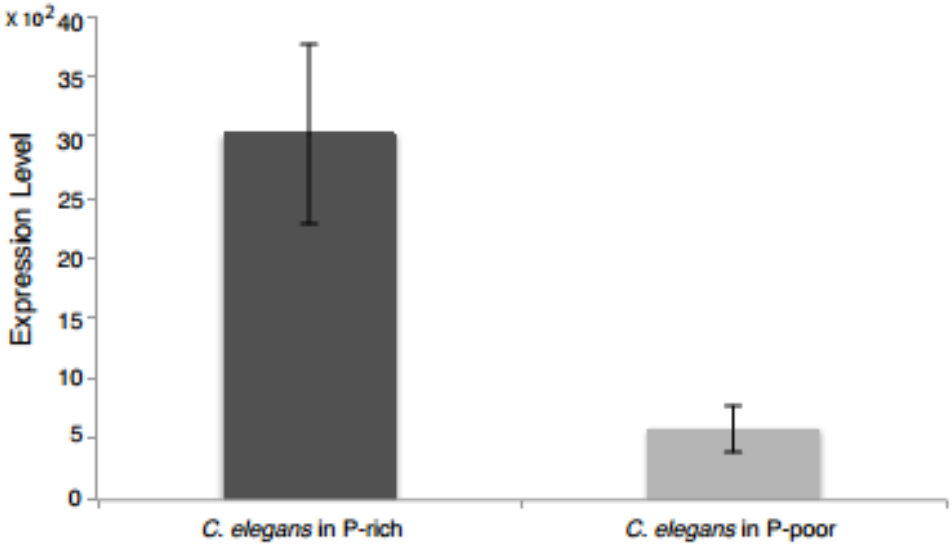

Figure 1. Expression Level of 18S rDNA in *C. elegans* reared in P-rich and P-poor media. ($P \leq 0.05$)

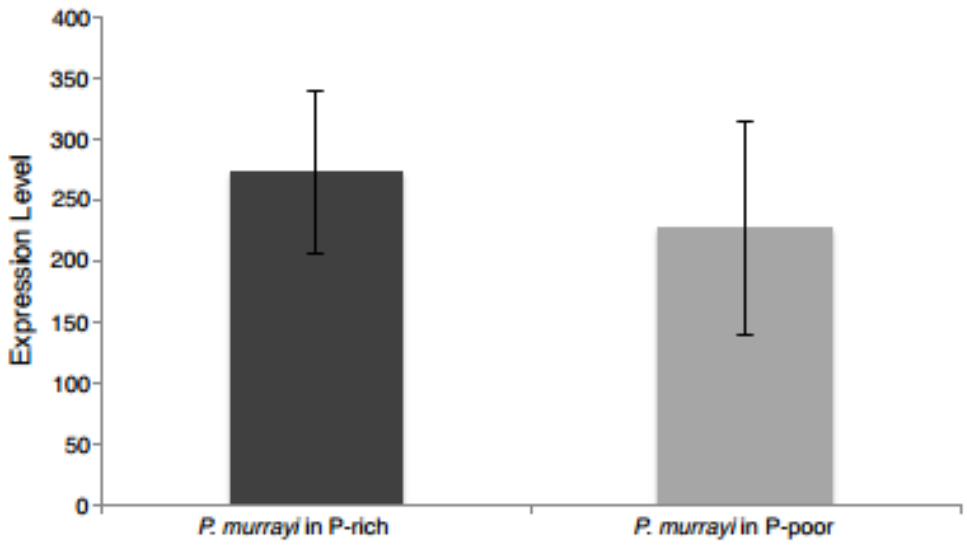

Figure 2. Expression level of 18S rDNA in *P. murrayi* reared in P-rich and P-poor media (P≥0.05)

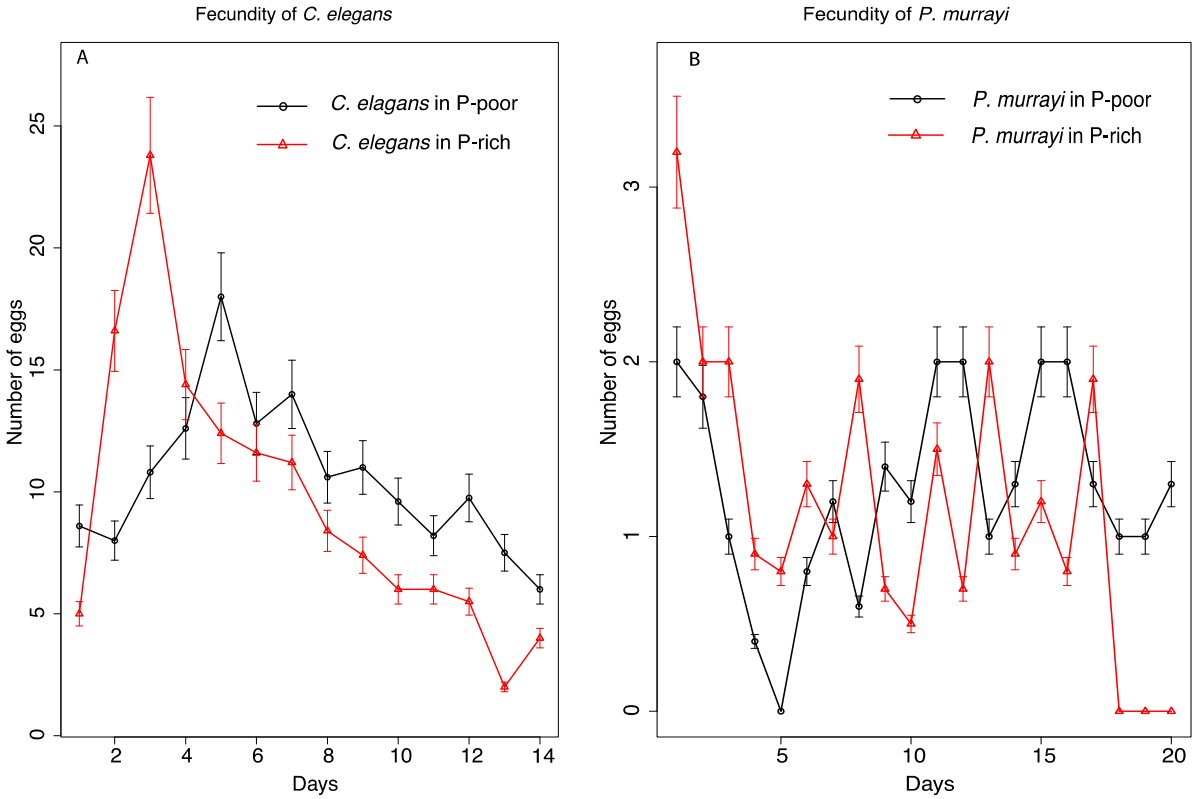

Figure 3. Fecundity of *C. elegans* (A) and *P. murrayi* (B) under P-poor and P-rich media. There was no consistent pattern for growth rate and fecundity.

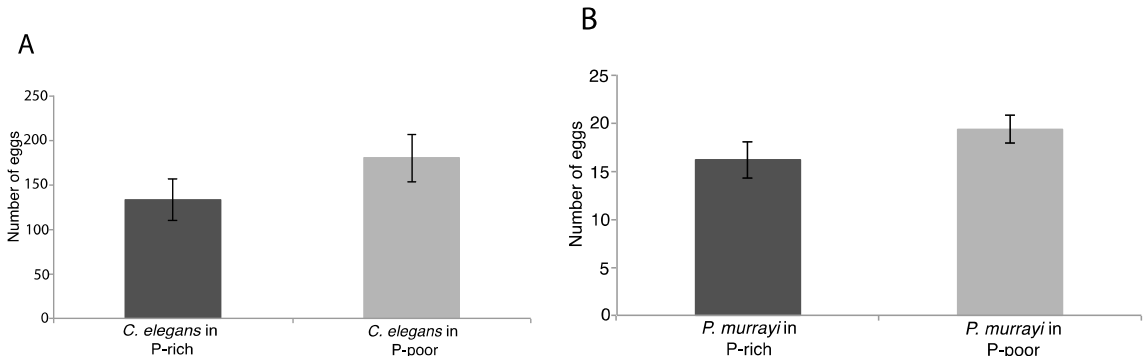

Figure 4. The total number of eggs produced by *C. elegans* (A) and *P. murrayi* (B) reared in P-rich and P-poor media ($P \geq 0.05$ for both of them). Both had a similar number of eggs regardless of different P media.

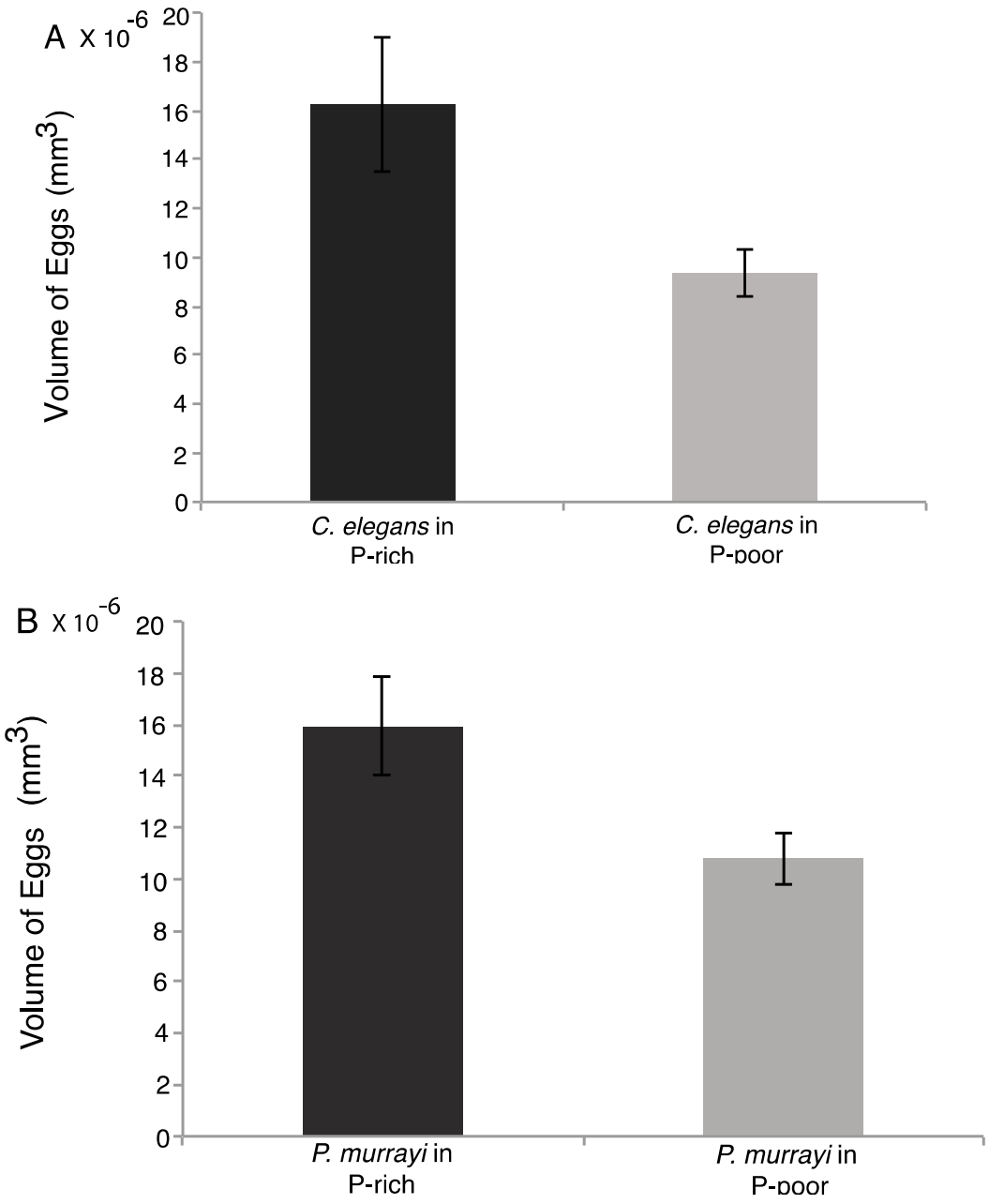

Figure 5. Average volumes of eggs from *C. elegans* (A, P= 0.034) and *P. murrayi* (B, P=0.04087) reared in P-rich and P-poor media.

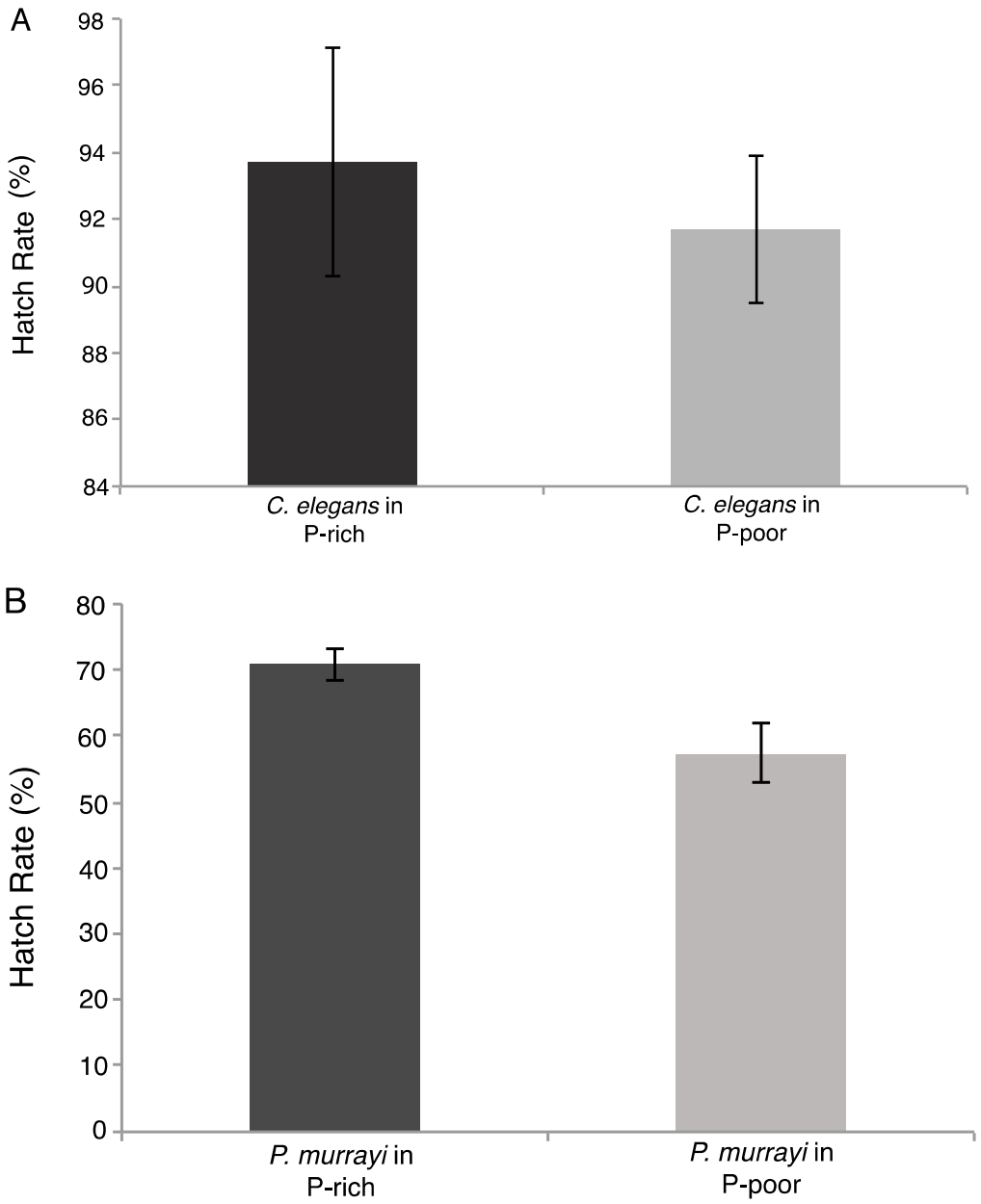

Figure 6. The hatch rates of *C. elegans* (A, P= 0.2062) and *P. murrayi* (B, P=0.04883) reared in P-rich and P-poor media

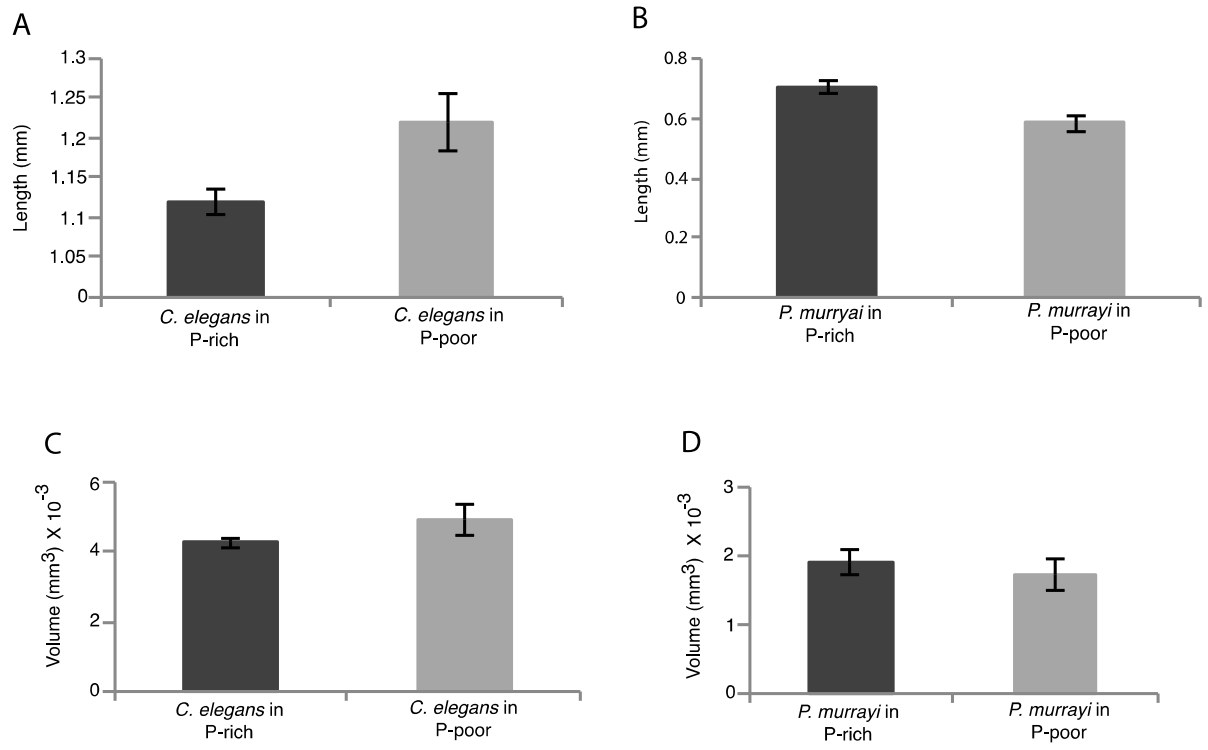

Figure 7. Body size of *C. elegans* and *P. murrayi*. A: Body length of *C. elegans* in P-poor conditions is longer than those cultured in P-rich ($P \leq 0.05$); B: Body length of *P. murrayi* under P-poor conditions is longer than in P-rich ($P \leq 0.05$); C: Body volume of *C. elegans* in P-poor conditions is smaller than those in P-rich ($P = 0.2056$); D: Body volume of *P. murrayi* in P-poor conditions is smaller than those in P-rich ($P = 0.08161$).

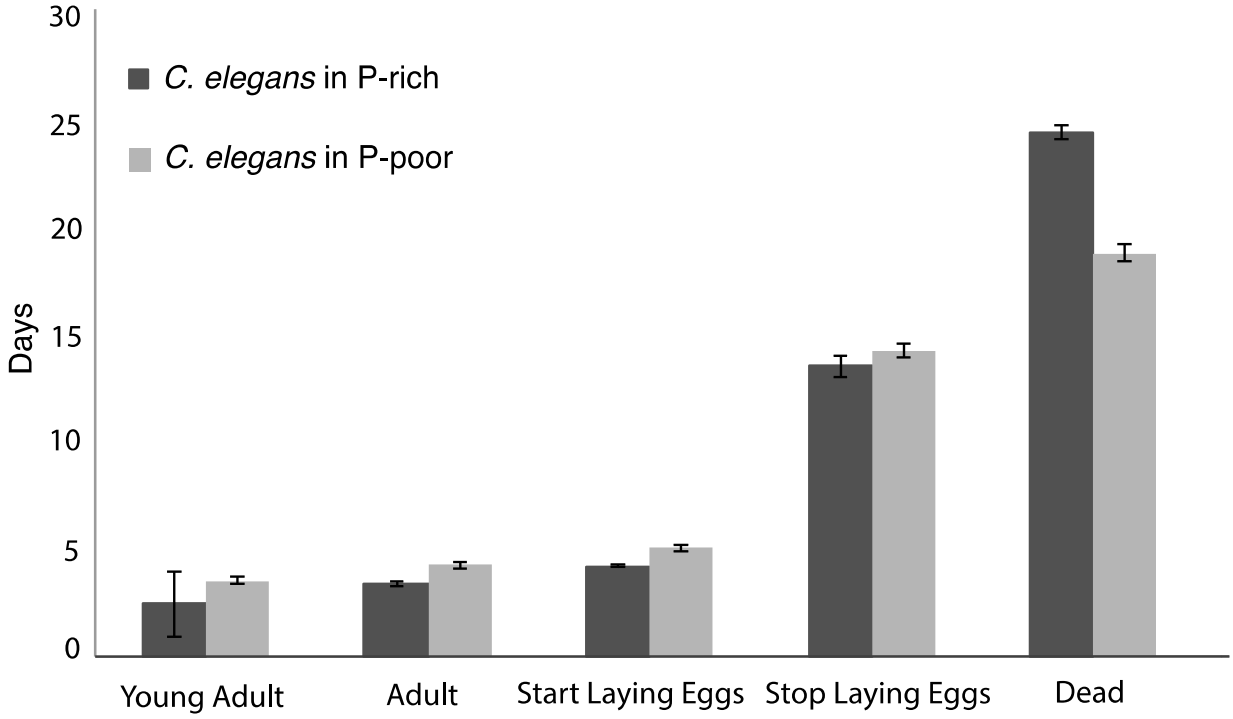

Figure 8. Comparison of the life history stages in *C. elegans* reared in P-rich and P-poor media. Time to young adult and death dates are significantly different at $P \leq 0.05$.

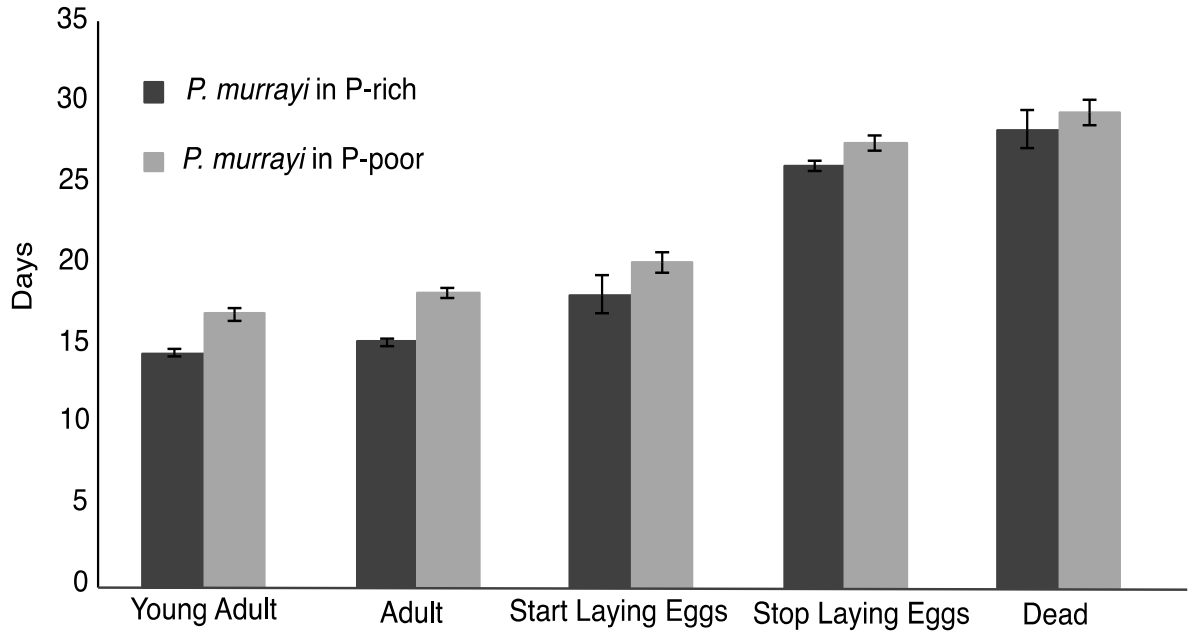

Figure 9. Comparison of the life history stages in *P. murrayi* reared in P-rich and P-poor media. Time to young adult, adult and start of egg laying dates are significantly different at $P \leq 0.05$.

Supplementary Figures and Tables

Figures

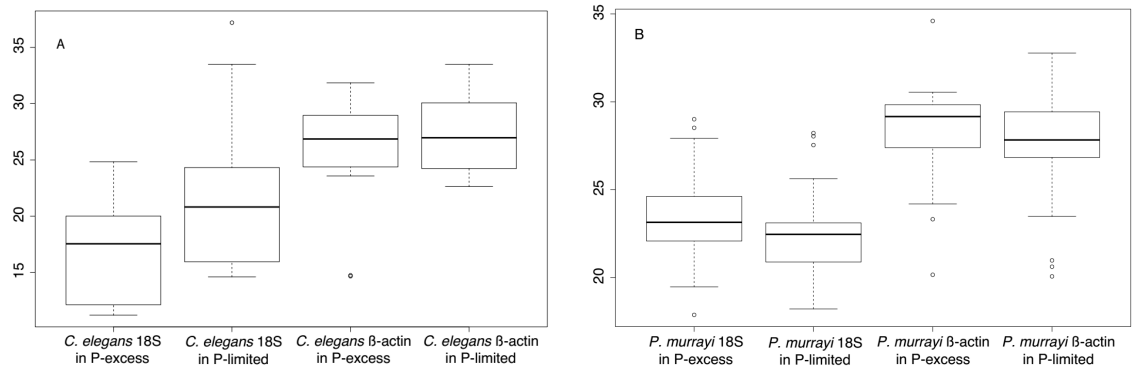

Figure S1. CT values distribution of *C. elegans* and *P. murrayi*

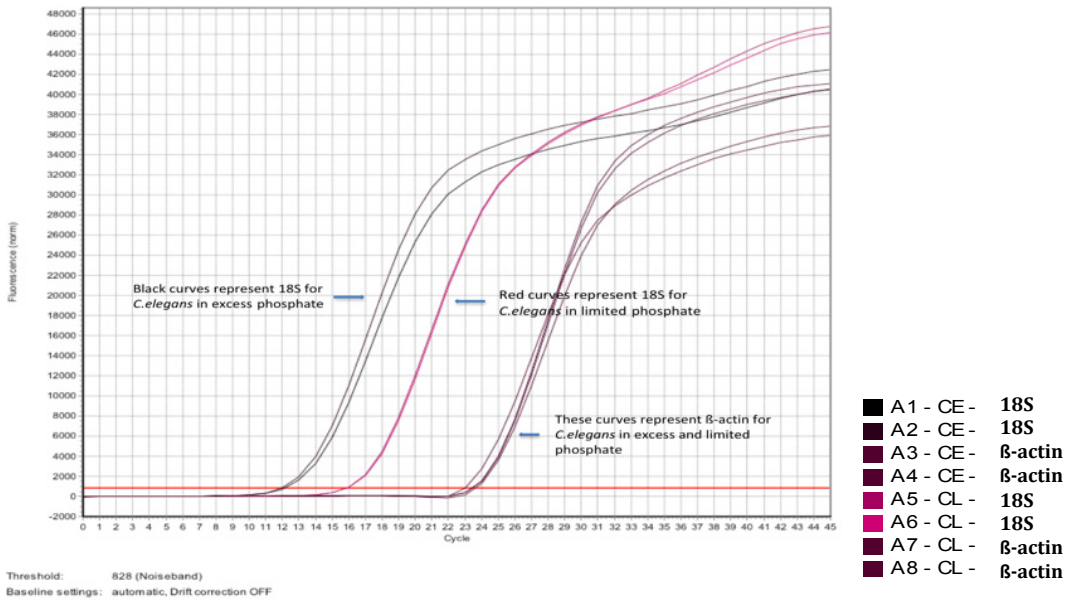

Figure S2. The CT values of 18S ribosome of *C. elegans* in P-rich and P-poor conditions.

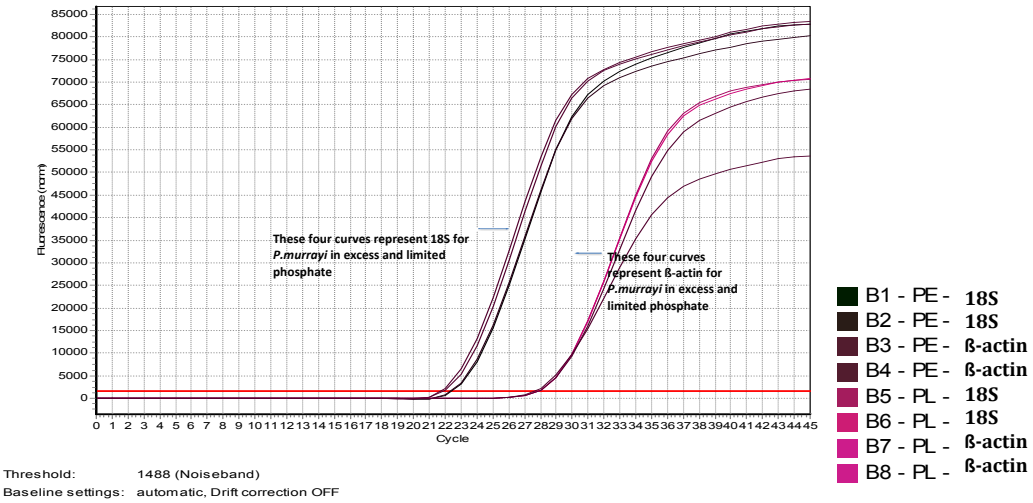

Figure S3. The CT values of 18S ribosome of *P. murrayi* in P-rich and P-poor conditions.

Chapter 3

Draft genome of an emerging model organism, *Plectus murrayi*

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Abstract

Plectus murrayi is one of the predominant invertebrates of Antarctic dry valley terrestrial ecosystems. Its unique physiology, phylogenetic position within Nematoda, and ability to be cultured on artificial media make *P. murrayi* an ideal model for exploring evolutionary responses to extreme environmental stress. Using whole genome and transcriptome sequencing we assembled a draft genome to test our hypothesis that persistence in an extreme environment can result in evolutionary changes that constrain genome architecture, including genome decay. A draft sequence analysis resulted in finding significantly less protein-coding genes, more exons, and fewer transposable elements than expected. Such reductions may be evidence of genome decay and intron retention, reflecting an evolutionary response to persistent and variable environmental stress. We discuss these findings in the context of the evolution of life history traits and stress tolerance.

Background

Free-living soil nematodes of the Antarctic McMurdo Dry Valleys (MDV) have persisted in one of the harshest terrestrial environments on Earth for millions of years (Convey et al., 2008). Freezing temperatures, limited nutrient supply, high osmotic gradients, low water availability and only brief periods of time each year when liquid water is available provides a backdrop of multiple environmental stresses to which these nematodes must respond and adapt (Doran et al., 2002; Fountain et al., 2014; Fountain et al., 1999).

As the top metazoan of most MDV soil food webs (Wall and Viginia, 1999), nematodes contribute to a wide range of soil processes and play an important role in soil nutrient cycling (Barrett et al., 2008). An understanding of Antarctic nematode genomes is a valuable first step towards identifying functional genes that are associated with stoichiometric ecological processes (Elser et al., 2000), but also environmental stress tolerance (Wharton, 2002), patterns of genome evolution and architecture (Nevo, 2001), and the resolution of important nodes in the nematode tree of life (Blaxter and Koutsovoulos, 2014).

Plectus murrayi is a free-living microbivorous Antarctic nematode that occurs in a wide range of soil habitats, reflecting a high dispersal capacity and broad ecological amplitude (Adams et al., 2014b; Velasco-Castrillón and Stevens, 2014). Unlike many organisms from extreme environments, *P. murrayi* can be cultured under laboratory conditions (Adhikari et al., 2010b), which provides sufficient numbers of individuals for genome sequencing without the potential for contamination by other species which can occur when genomic libraries are prepared from environmental samples. Under laboratory conditions at 15 ˚C its egg-to-egg lifecycle is 53-57 days (de Tomasel et al., 2013). Given that austral summer temperatures provide only a few days above freezing each year, most individuals require multiple years of

exposure to intracellular freezing, thawing, and desiccation in order to carry out their entire lifecycle. In response to these environmental stressors, *P. murrayi* has evolved molecular genetic mechanisms to facilitate anhydrobiosis (Adhikari and Adams, 2011; Adhikari et al., 2009) and freezing tolerance (Adhikari et al., 2010c). Thus, it has been suggested that *P. murrayi* is an excellent emerging model organism for studying the origin and evolution of metazoan stress response mechanisms (Adhikari et al., 2010a).

Genome decay (gene loss and genome reduction) is a phenomenon known to occur widely in prokaryotes (Couce et al., 2017; Judelson et al., 2012; Mandadi and Scholthof, 2015) and eukaryotes, perhaps the best examples of this being the loss of significant amounts of genomic and morphological complexity by Tardigrada (Smith et al., 2016) and Dicyemids (Kobayashi et al., 1999) but examples can be found throughout Nematoda as well (Mitreva et al., 2005). Such reductions are thought to arise from selection pressures associated with deletion bias (Mira et al., 2001), simplification (Smith et al., 2016) or increasing specialization (Cramer et al., 2011; Smith et al., 2006), including parasitism (e.g. Kobayashi et al., 1999). We hypothesized that in response to glacial cycling from the quaternary to the present (Convey et al., 2009), and corresponding changes from biotic to primarily abiotic drivers of fitness, *P. murrayi* has repeatedly lower the expression of some genes that became unnecessary or costly to maintain, such as those involved in competing for similar resources or evading predation. Makarova (Makarova et al., 2006) and his colleagues show that there are some unnecessary genes in lactic acid bacteria lost functions, in order to adapt to new culturing condition, such as the genes related to sugar PTS transporters and metabolism. Increasing number of evidence have support that genome decay occurs frequently in microbes, there is a slightly deleterious mutation was found in Escherichia coli (Balbi et al., 2009), and some studies also found unnecessary genes

absent in pathogens, such as genes encoding flagellar in Hyaloperonospora arabidopsidis (Judelson et al., 2012), gene loss in bacterial symbionts (Moran, 2003).

P. murrayi is found only in the limno-terrestrial environments of continental Antarctica where it persisted for thousands, if not millions of years (Convey et al., 2008; Convey et al., 2009; Pugh and Convey, 2008). Among the coldest, driest, windiest ecosystems on earth, the diversity and functioning of these ecosystems are strongly driven by physical controls (Fountain et al., 1999; Vincent, 2000). Food webs in these ecosystems are simple (Convey 2013), structured influenced by relatively few biotic interactions (e.g. predation and competition) (Hogg et al., 2006). Although *P. murrayi* inhabits a highly variable environment in terms of environmental extremes (temperature, moisture/desiccation, solar radiation, osmolarity (Courtright et al., 2001), we considered that an adaptive response to an increasingly austere environment would entail selection for the maintenance of fewer, but more specialized genes, and strong selection against maintenance of genes that are advantageous only in more complex ecosystems. In this study, we set out to explore the genome assembly and gene expression of *P. murrayi*, the first metazoan from continental Antarctica with a complete, albeit draft, genome sequence, and try to establish a foundation to learn its adaptive responses to surviving in an increasingly simplified ecosystem.

Specimen collection and maintenance

Populations of *P. murrayi* were collected from Antarctic Dry Valley soils from 2008-2009 and maintained in the laboratory on phosphorus sand agar media at 15 ˚C according to Adhikari et al., 2010. The final media consisted of 15g agar, 965 ml H_2O, 20 ml BMB (Bold Modified Basal), and 10.33 mg K_2HPO_4. The pH was adjusted to 7.0 and then H_2O was added to 1.0 L, and the mixture was autoclaved for 20 minutes at 120 ˚C. Sterile Sand was poured on

cooled plates, which were stored at 4 ˚C. 30µL of stock *E. coli* OP-50 was added to each plate using a cotton swab. The OP50-inoculated plates were incubated at 37˚C for 2 days. After the two-day incubation period, nematodes were added to the plate and incubated at 26˚C for 1 week, followed by incubation at 15˚C. For keeping our nematode cultures in health and fresh condition, we transfer a piece of agar with nematode on the new plates every 3 weeks.

However, there are some troubles in our study, the nematode samples from Antarctica is hard to collected and gathered to a massive amount for genome sequencing, and the artificial culturing bring some contamination from bacteria that *P. murrayi* has been fed on.

DNA extraction and library preparation

Populations of *P. murrayi* were washed and rinsed three times in 0.4 % Hyamine, and kept in Ringer's solution for 15-30 minutes before DNA extraction. Wizard Genomic DNA Purification Kits (Promega, Madison, WI) was used for extracting DNA, and the genomic DNA was treated with RNase A to remove any RNAs in the sample. The genomic library was constructed using an Illumina Paired-End DNA Sample Preparation Kit following the manufacturer's instructions. Genomic libraries were sequenced on an Illumina 2000 Genome Analyzer IIx sequencer in paired-end mode with the read length of 76 bp. Because of the difficulties to gathering large amount of *P. murrayi*, the library build in a low concentration total DNA and needs to do a necessary amplification.

Genome assembly and Annotation

Sequence reads were trimmed in Trimmomatic (V 0.36) (Bolger *et al.* 2014) and assembled with SOAP de novo (Luo *et al.* 2012), 67-mer was calculated by KmerGenie (Chikhi & Medvedev 2014). EVidenceModeler (EVM), and Augustus 2.2.5 (Stanke et al., 2008) were used to integrate *ab initio* gene predictions with *P. murrayi* RNAseq data taking into account

transcript assemblies (done with PASA47) and protein homology (Haas et al., 2003). Blast2GO analysis was also used for mapping and annotation (Götz et al., 2008). The assembled genome was converted to NCBI format and assigned as NCBI bioSample project number SAMN04625768.

We obtained 107,770,211 raw reads, with an average insert size of 550 bp. The draft genome assembly of *P. murrayi* is 238 Mbp with an N50 length of 5,274 bp, maximum scaffold size of 270,881 and G+C content of 44.50%. Sequencing quality statistics and genome assembly information are shown in table 1. After we cleaned up the raw reads and assembled them with an appropriate K-mer, the N50 of our assembly is over 5000 with coverage around 340×. A relative large number of scaffolds of our genome made us to trim the shorter ones before we did the annotation and gene prediction. In the further research on *P. murrayi*, we hope to have better way to prepare the library and sequence it with limited number of samples.

Compared to *C. elegans, P. murrayi* (table 3) has less number of genes, but a larger genome size, shorter exons, and fewer transposable elements (table 4) (Spieth et al., 2014). Meaningful inferences about gene and genome evolution in *P. murrayi* will require comparisons of more closely related taxa methods using phylogenetic comparative methods (Brooks and McLennan, 1991; Harvey and Pagel, 1991), but we speculate below.

Distribution of alternative splicing modes

The annotation file of *P. murrayi* (gtf file) was obtained from Augustus 2.2.5. In order to compare *P. murrayi* to *C. elegans* and other organisms, we downloaded the annotation file of *C. elegans* from wormbase (ftp://ftp.wormbase.org/pub/wormbase/releases/WS253/species/c_elegans/PRJNA13758/). We

performed an alternative splicing transcriptional landscape analysis on the two species using the AStalavista web server (http://genome.crg.es/astalavista/).

As mentioned above, the genome size of *P. murrayi* is smaller than *C. elegans*, while the number of genes in *P. murrayi* than in *C. elegans*. To understand the specific gene coding strategy, we examined their strategy of coding genes and the distribution of their alternative splicing modes are shown in table 5.

The distribution of alternative splicing modes of *P. murrayi* and *C. elegans* shows the percentage of intron retention is 20.67% and alternative exon is 22.94% in *P. murrayi*, while the percentage of intron retention is 14.31% and alternative exon is 20.15% in *C. elegans*.

According to gene prediction and annotation by Augustus (Stanke et al., 2008), PASA (Haas et al., 2003) and EVM (Haas et al., 2008), the total number of protein-coding genes is 14,680, including UTRs, and Exons (table 2). The gene ontology (GO) term and pathway analysis by Blast2GO (Götz et al., 2008) is presented in Figure 1 and Figure 2.

Compared to *C. elegans*, the genome of *P. murrayi* contains relatively fewer sequences associated with behaviors and locomotion, and more that involve metabolism and cellular processing. This observation is consistent with an evolutionary response to increased demand for responding to physical abiotic drivers, as well as decreased demand for gene products involved in biotic interactions, such as competition and predation (Convey, 1996; Hogg et al., 2006). However, since the majority of recovered sequences are of unknown function, inferences requiring comparisons of the relative frequency of genes involved in responses to biotic and abiotic stresses are speculative.

We predicted functional genes of *P. murrayi* using the genome of *C. elegans* as reference species and compared gene expression of RNA-seq data of *P. murrayi* to *C. elegans*, and in the

top 50 most significant differently expressed genes, we found 49 genes down-regulated in *P. murrayi*.

Based on the most complete phylogenomic analysis of the Nematoda to date (Blaxter and Koutsovoulos, 2015; Koutsovoulos, 2015), *P. murrayi* is the sister taxon to the Secernentea, and as such plays a key role in determining character polarity and understanding the evolutionary radiation of the Secernentea. The Secernentea is the lineage that produced *C. elegans* and virtually all of the major plant and animal parasitic clades of nematodes, many of which are scientifically and economically important, and/or serve as model species for molecular, developmental and genetic studies (Adhikari et al., 2009; Coolon et al., 2009; Gao et al., 2008; Kim et al., 2014).

Since *P. murrayi* tolerate a number of environmental stresses from Antarctica and occurs in a wider range of soil habitats (Adams et al., 2014a), establishing a genomic database of *P. murrayi* makes it a perfect novel model organism for understanding the evolutionary messages of organisms, such as genome evolution, intron retention, genes adaptation in extremely harsh environment, and survival strategies under multiple and strong environment stresses.

Conclusion

Genome decay is known to occur for organisms evolving in response to environment stress (Faddeeva-Vakhrusheva et al., 2016; Marguerat et al., 2014), increased use of alternative splicing could be a way for organisms to generate additional gene products and mitigate the loss of functional genes (Ma et al., 2018; Mandadi and Scholthof, 2015). We found the total number of genes of *P. murrayi* is relatively larger than *C. elegans* and the intron retention percentage is twice as much in *P. murrayi* than in *C. elegans* (table 5), which suggests the intron sequences also could be used for coding functional genes when there are limited numbers of exons due to

environmental stress. To adapt to harsh environment and use of energy in a smart way, *P. murrayi* reduce its genome size and use introns more efficiently to coding functional genes with smaller number of exons in elemental resources limited environment (Filichkin et al., 2018; Schmitz et al., 2017). Some studies found intron retention occurs commonly by environmental stress (cold or heat) in plants (Filichkin et al., 2018). Furthermore, intron retention can be used for coding proteins when the number of coding genes is lacking, and make more hypothetical genes with unknown functions, some of these genes could also be a part of numbers of biogenesis and metabolism processes (Kalinina et al., 2018; Palud et al., 2018; Pang et al., 2017; Peter et al., 2011).

Among the top-50 differentially expressed genes between *P. murray* and *C. elegans* (Figure 4), only histone synthesis associated genes (His-39) are up-regulated in *P. murrayi*, which is related to DNA binding and protein heterodimerization activity and conserved in zebrafish, mosquito and some mammals (Rodríguez-Aznar et al., 2013). Most of the genes in *C. elegans* that are upregulated compared to *P. murrayi* are related to metabolism and development, such as cell division and cell membrane building, which suggest the development and reproduction of *C. elegans* are faster than them of *P. murrayi* in our cultured condition. Among the functional genes shared between *C. elegans* and *P. murrayi*, we found that *Lea-1* and *daf-16* are down-regulated in *P. murrayi* while heatshock genes and ubiquitin protein coding genes up-regulated in *P. murrayi* relative to *C. elegans*., each of which are implicated in stress tolerance in nematodes (Bond and Schlesinger, 1985; Deegenaars and Watson, 1997; Gillan and Devaney, 2014; Labbadia and Morimoto, 2015; Rinehart et al., 2006; Sugi et al., 2011). This observation is consistent with the hypothesis that in response to constant threat of exposure to environmental extremes, *P. murrayi* constitutively expresses many genes, particularly those involved in

environmental stress survival (Adhikari and Adams, 2011; Adhikari et al., 2009, 2010c).However, this results also shows not all the genes related to stress tolerance are up-regulated in *P. murrayi*, which might because we cultured both *P. murrayi* and *C. elegans* on the media and temperature that is better for *P. murrayi*, so *C. elegans* population were grown under stressed condition and some stress tolerance related genes up-regulated in them.

By comparing the top 50 differentially expressed genes between *P. murrayi* and *C. elegans* under controlled environmental conditions we found decreased expression of some important functional genes in *P. murrayi*, including *unc-16* (essential gene for normal synaptic transmission) (Byrd et al., 2001); alh-6 (Protein for aldehyde dehydrogenase); *nfm* (neurofibromatosis homology gene); *pat-2* and *cdc-42* (cell division control genes). *cdc-42* is a mediator of engulfment signaling that can regulate downstream of integrin-alpha pat-2 (Neukomm et al., 2014), moreover, high expression of *cdc-42* mostly occurs around apoptotic cell corpses . Other important genes there were downregulated in *P. murrayi* include *tnt-3* (calcium ion binding), *klp-12* (kinesin-like protein for ATP binding) and *Lea-1* (late embryogenesis abundant) which plays a role in survival under environmental stresses, such as desiccation and heat (Gal et al., 2004). Also downregulated in *P. murrayi* is *sir-36* (detection of chemical stimulus) and *ufd-1*, which is a key member of *cdc-48* complex that participates in endoplasmic reticulum (ER)-associated protein degradation (Mouysset et al., 2008), which regulates DNA replication, we also found Gln-3 was down-regulated in *P. murrayi* compare to *C. elegans*, which is involved in nitrogen compound metabolic process regulation. Averagely, most of genes related to metabolism and cellular processes in nematodes are up-regulated in artificial cultured *P. murrayi* compared to them live in the wild (figure 5), suggests that the more genes

would be coded and expressed when there are more available nutrients from environment compared to limited life-support habitat in Antarctica.

Researchers have suggested that variation in organismal stoichiometry is related to the availability of nutrients (e.g., nitrogen and phosphorus) in the environment (Elser et al., 2000; Sterner and George, 2000; Zimmerman et al., 2013). Mechanisms underlying the observed variation in biological stoichiometry can be coordinated by a combination of genetic and environmental drivers (Carroll et al.; Mathis et al., 2017). C : N : P stoichiometry from environment will effect on those genes related to development of nematodes and influence on the trophic interaction, which can feedback on nutrient cycling in the environment (Elser et al., 1996; Elser et al., 2000; Sterner and George, 2000). In nitrogen limited environment, organism might lower the expression of some genes and use available nitrogen more efficiently (Elser et al., 1996). Some genes associated with ATP binding and ATPase activity were down-regulated in *P. murrayi*, it suggests that genes related to development and metabolism might reduce their expression to adapt to C, N, P limited environment. Because *P. murrayi* grows more slowly than *C. elegnas*, the usage of available nutrients in *P. murrayi* might very conserved as it adapts to Antarctica acrid soils for a long time.

Assembled and annotated the genome of *P. murrayi* starts a new work frame of Antarctic nematodes and even the whole community there. Due to Antarctic extremely harsh and nutrient-limited environments, the specific adaptation strategies of them are valuable to study at genetic level, it will provide more evidence to reconstruct high resolved phylogenetic tree among Antarctic nematodes. Moreover, by mapping these genes onto the highly resolved phylogenetic tree, we could find out those stress tolerant genes evolutionary pattern in Antarctica, such as duplication event or gene loss and gain events.

Availability of supporting data

The draft genome sequence has been deposited in GenBank under the accession LZQM00000000. The version described in this article is version LZQM00000000.

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Author's Contributions

XX, RAD and BJA designed this study. BJA collected the nematode samples, ARD prepared and sequenced the raw reads. XX assembled and annotated the genome, AS and SF were involved in data analysis. XX and BJA drafted the manuscript and all other authors contributed significantly to subsequent drafts. All authors read and approved the final manuscript.

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Figures and Tables

Figures

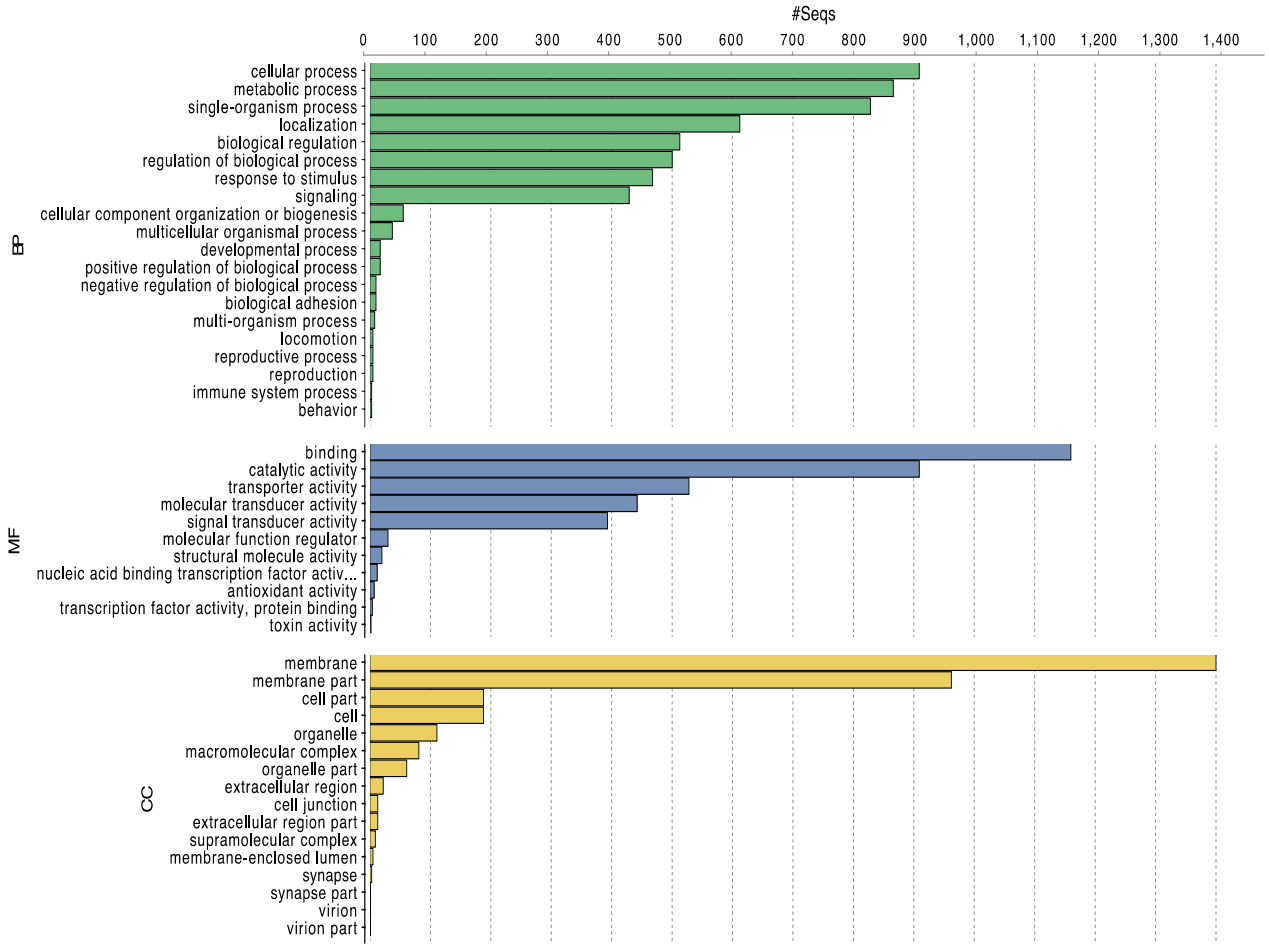

Figure 1. Gene ontology distribution of *P. murrayi* annotation from Blast2GO.

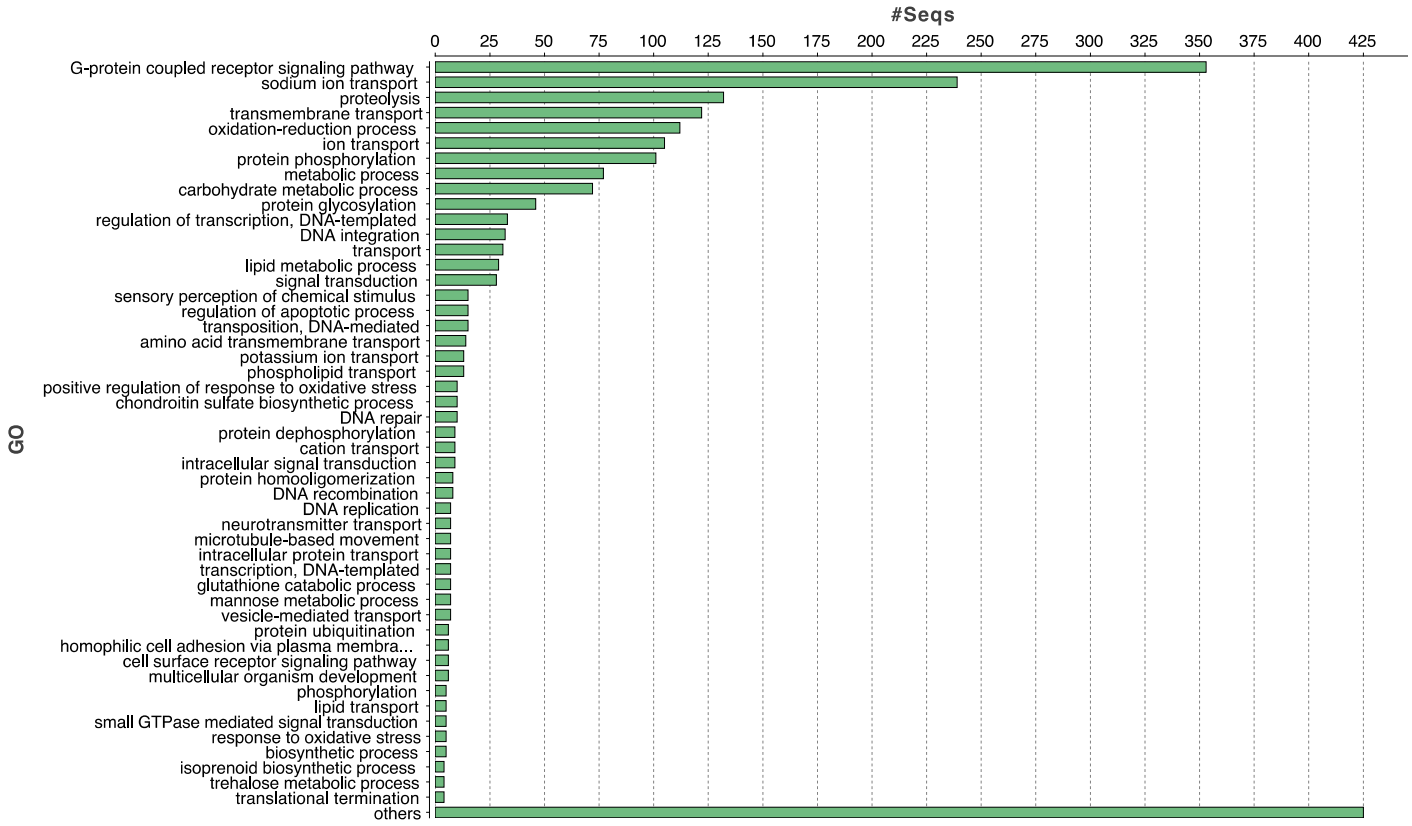

Figure 2. Gene ontology counts of the genome of *P. murrayi*.

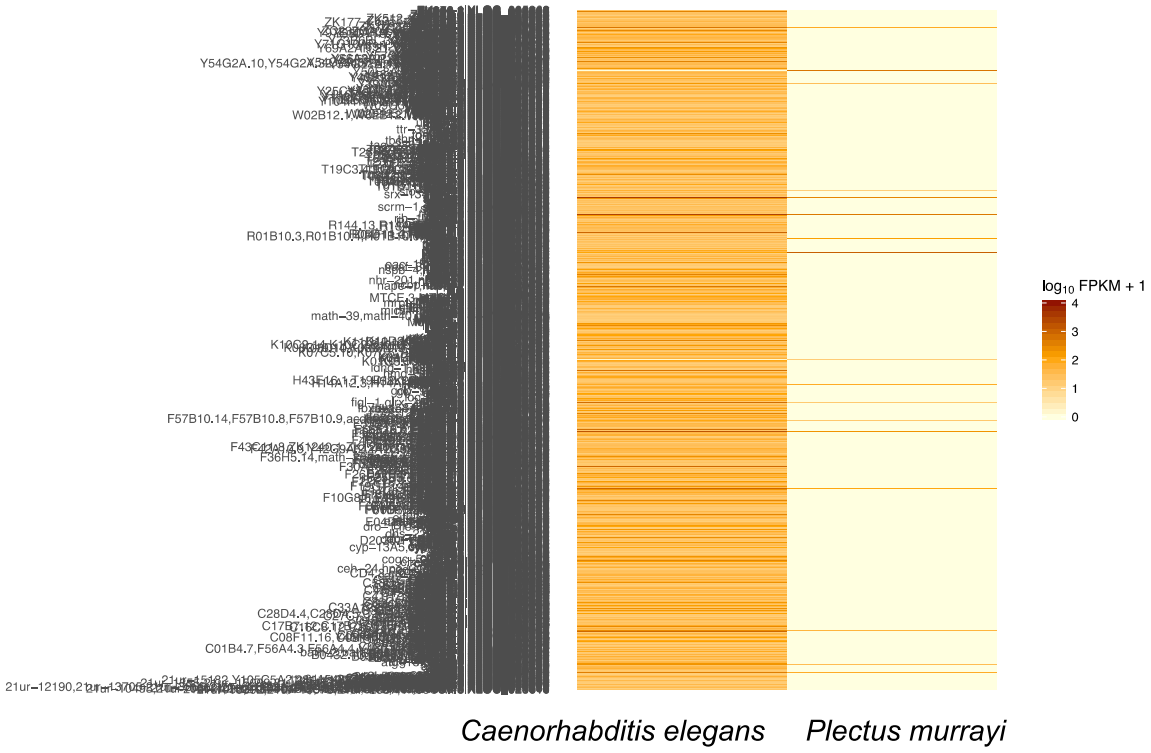

Figure 3. Significantly different expressed genes of *C. elegans* and *P. murrayi*

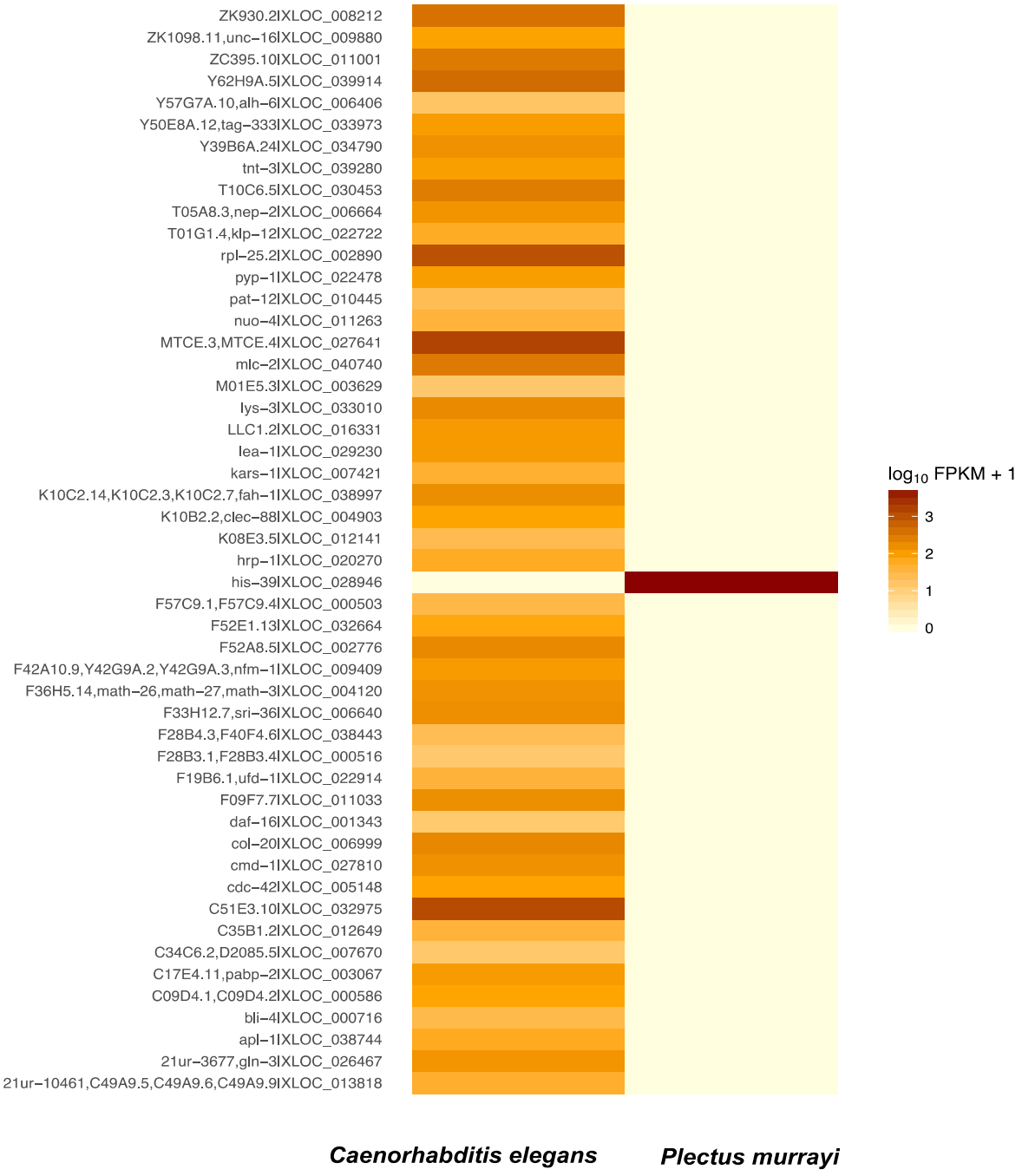

Figure 4. Top 50 significantly different expressed genes of *C. elegans* and *P. murrayi*, from a comparison between RNA-seq data between *C. elegans* and *P. murrayi* at 15 ˚C with regular Phosphorus supply.

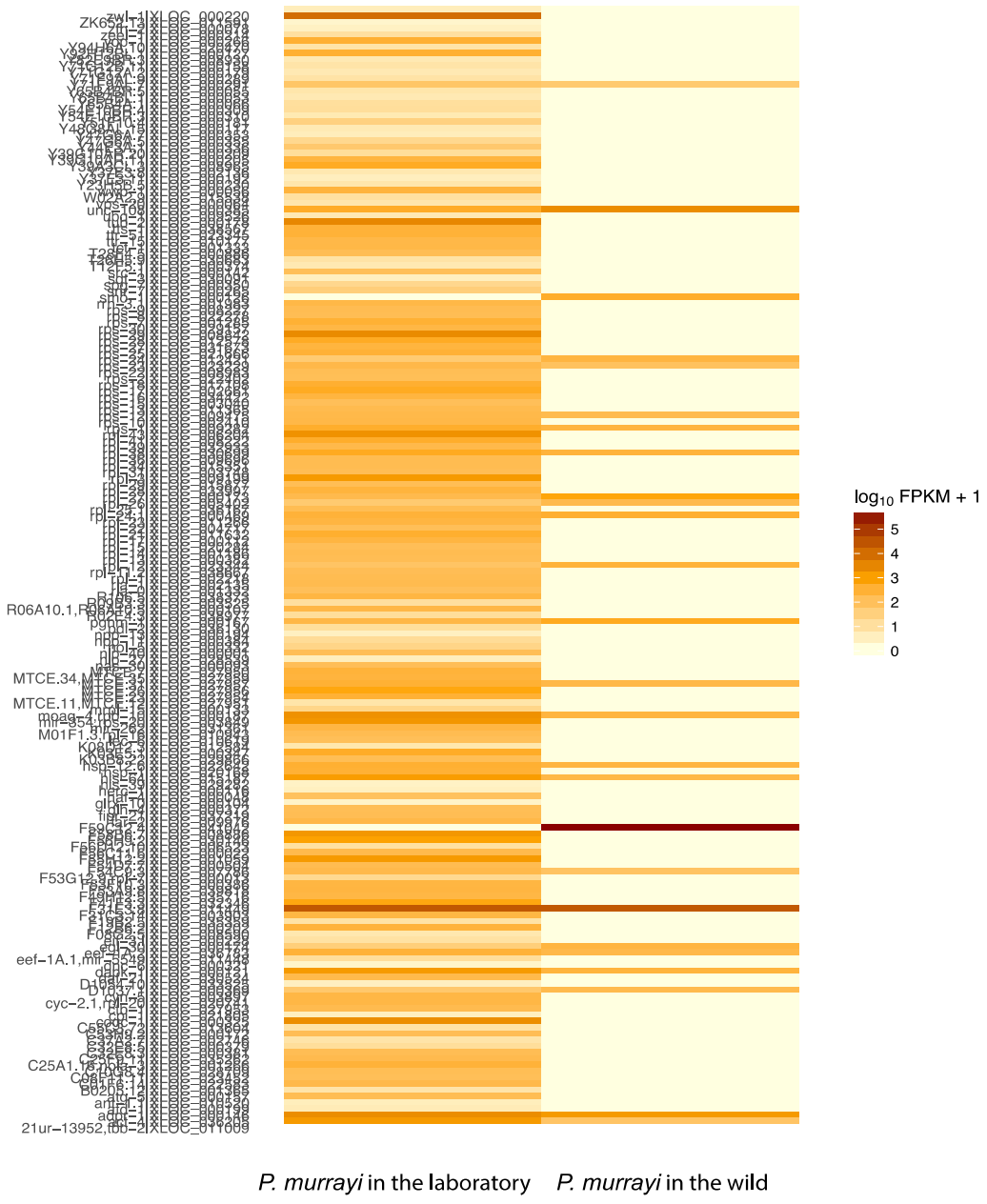

Figure 5. Top 100 significantly different expressed genes of *P. murrayi* in the laboratory and in the wild.

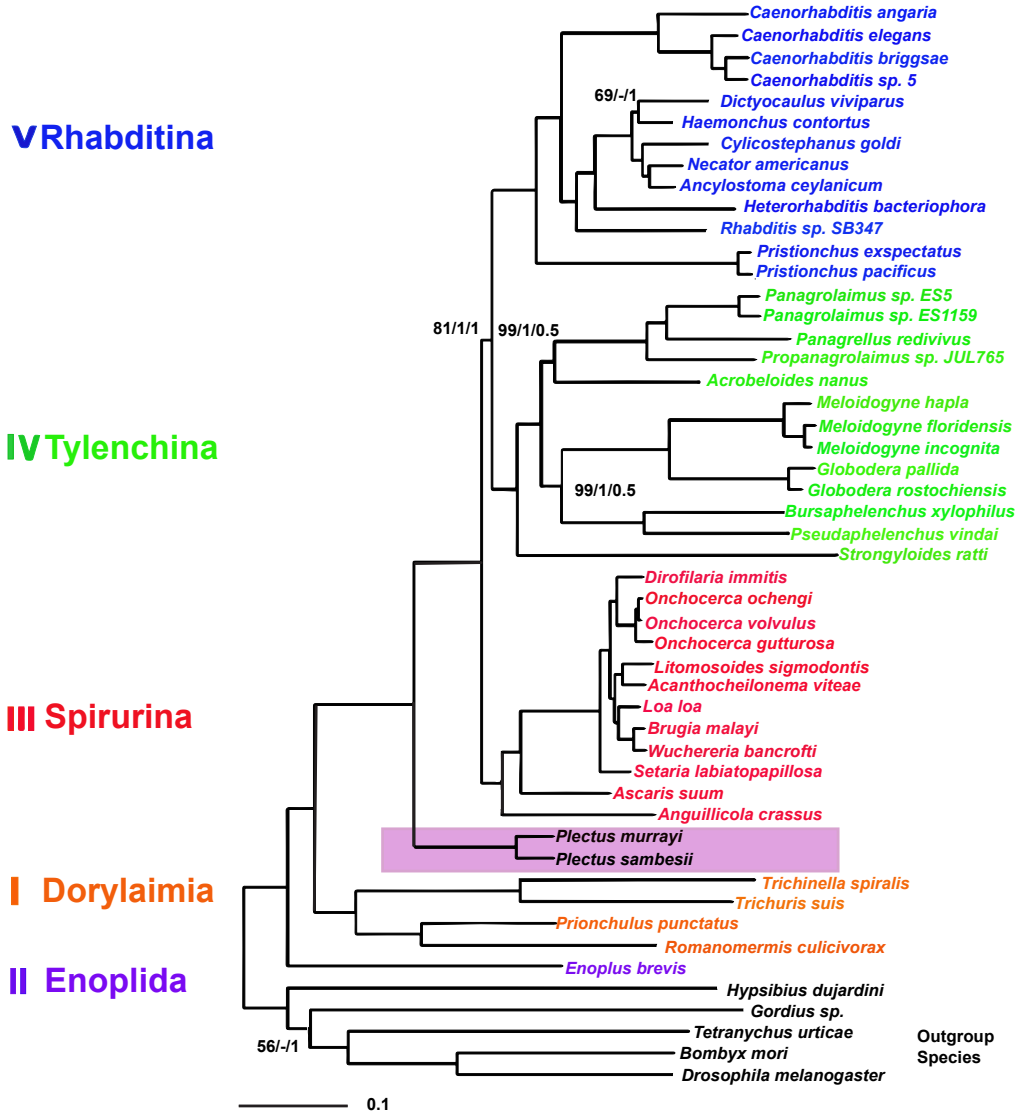

Figure 6. Nematode phylogeny modified from Koutsovoulos's work (Koutsovoulos, 2015). RAxML bootstraps values below 100 or PhyloBayes posterior probabilities below 1 are shown on the nodes.

Tables

Table 1. *P. murrayi* Draft Genome Summary

Assembly size (bp)	238,314,937
N50	5,274
N50 length (bp)	12,384
G+C (%)	44.50
Gene density (gene/Mb)	250.21

Table 2. *P. murrayi* Gene Summary

Genes: EVM	
Total number of genes	14,680
Mean gene length(bp)	1641.809
Exons: EVM	
Number of exons	139,736
Mean number per gene	9.52
Mean length(bp)	257.179
Introns: EVM	
Number of introns	153,487
Mean number per gene	10.46
Mean length(bp)	351.46
UTRs: PASA	
Number of genes having UTR	116,51
Mean UTR length(bp)	64.182
Number of 5'UTRs	108,89
Mean 5'UTR length (bp)	21.628
Number of 3'UTRs	11,365
Mean 3'UTRs length(bp)	104.955
Number of mRNA	59,649
Number of transcript	59,649

Table 3. *P. murrayi* gene structure compare to *C. elegans*

Genes: EVM	*P. murrayi*	*C. elegans*
Genome size	260 million bp	100 million bp
Total number of coding genes	14,680	20,222
Mean gene length(bp)	1.64k	1.83k
Number of exons	139,736	125,702
Mean number per gene	9.52	5
Mean length(bp)	257.197	1.0k

Table 4. Soft mask on assembled genome of *P. murrayi*

P. murrayi	Number of elemens	Lenghth Occupied
Retroelements	262 (0.02%)	412,812 bp
DNA transposons	6,071 (0.20%)	481,292 bp
Samll RNA	0	0
Satellites	310 (0.01%)	18,394 bp
Simple repeats	3,593 (0.13%)	319,399 bp
Total interspersed repeats		642,623 bp (0.27%)

Table 5. Alternative splicing model of *P. murrayi*, *C. elegans* and *Drosphila yakuba*

	Alternative acceptor	Intron Retention	Alternative donor	Exon skipping	Other Events*
Plecuts murrayi	20.54%	20.67%	15.45%	22.94%	20.4%
Caenorhabditis elegans	26.17%	14.31%	15.80%	25.60%	18.12%
Drosophila yakuba	25.13%	12.43%	22.12%	20.15%	20.17%

Chapter 4

De novo transcriptome assemblies of Antarctic free-living nematodes, *Plectus murrayi* and *Scottnema lindsayae*

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Abstract

Background: Antarctic Dry Valleys, the driest and coldest deserts on earth, is a unique habitat with extremely low biodiversity because of their harsh environmental condition. This environment has extremely limited available water and nutrients with low tamperature, and nematodes play an important role in nutrient cycling and occupy the top of the food web in this desert. Both *Scottnema lindsayae* and *Plectus murrayi* are endemic nematode species that occur in different types of soil habitats in Antarctica, although *S. lindsayae* prefers dry, salty soils, while *P. murrayi* occurs more frequently in wet soils with higher available C, N, P and lower salinity. To improve our understanding of the nematode phylogeny and the mechanisms that allow these species to survive in dry, cold environments where nutrients are exceedingly limited, we sequenced and assembled transcriptomes for both of *S. lindsayae* and *P. murryai*.

Findings: Trinity de novo assemblies with Trinity-de novo (V 3.1.1) assembler and trinotate was used to predict the annotation for both of *Scottnema lindaysae* and *Plectus murrayi*. Raw reads were obtained from illumine hiseq 2500 single-end sequencing, and total transcripts of *S.*

lindaysae and *P. murrayi* are 29,402 and 31,165, respectively. The annotated genes of *S. lindaysae* and *P. murrayi* are 13,236 and 12,331, respectively. TransRate and Bench-marking universal single-copy orthologs analyses shows over 75% mapping rates and which indicated quality assemblies and a high degree of completeness for both *S. lindaysae* and *P. murrayi*. Conclusions: The transcriptomes of these two Antarctic nematodes establish a reference dataset for studying bioinformatics of other nematodes survive in high environmental stress habitats, and they are the only Antarctic nematode species have been sequenced up to date. Comparison between the transcriptomes of *Scottnema lindsaya*e and *Plctus murrayi* might also provide evidence that can reveal the factors that drive their distributions in Antarctic soil ecosystems. According to their different distribution, we found that the genes related to environmental stress tolerance expressions are different in *S. lindsayae* and *P. murryai*, which provides more evidence for their adaptation in specific habitat with genetic evolution.

Background

Antarctic nematodes survive in the driest and coldest desert in Antarctic dry valleys and tolerate various environmental stresses, such as freezing temperature, desiccation, sharp freeze thaw cycles (Wharton and Raymond, 2015) and oligotrophy (Campbell et al., 2013). Antarctic soil ecosystem has much lower biodiversity and are less productive nutrients cycling compared to other soil ecosystems (Wall and Viginia, 1999), and because of the scarcity of primary producer, nutrient cycling is more complex among the consumers that live there, most of consumers also participate in decomposition and nutrient cycling to obtain necessary energy to survival (Barrett et al., 2007; Cannone et al., 2008; Fritsen et al., 2000). Nematodes occur on every continent, including in Antarctica(Adams et al., 2006a; Adams et al., 2014b; Bamforth et al., 2005; Velasco-Castrillón et al., 2014), and are the top predator of the food web in this unique and harsh soil ecosystem (Raymond et al., 2013). *Scottnema lindsayae* is the nematode species have largest abundance from the McMurdo Dry Valleys (Courtright et al., 2001; Freckman and Virginia, 1993; Freckman and Virginia, 1997), and was described from Victoria Land in 1971 (Boström et al., 2010). *S. lindsayae* is mostly found in salty and dry soils at a wide range of elevations from the McMurdo Dry Valleys to about 1300 m above sea level (Adams et al., 2014b; Powers et al., 1998). This species is not commonly found in large populations in moister habitats near streams and lakes (Adams et al., 2014b; Courtright et al., 2001; Freckman and VIRGINIA, 1997). Moreover, *Plectus murrayi* is the most abundant *Plectus* nematode from the McMurdo Dry Valleys, and they occur more frequently in soils with higher moisture and sediments (Adams et al., 2006b; Ayres et al., 2007).

In Victoria land, *S. lindsayae* usually found in the soils near south shore of Lake Hoare (Figure 1) (Powers et al., 1998), which is arid and sandy with lower moisture, higher salinity (4100 mS cm^{-1}) and pH (Courtright et al., 2001; Freckman and Virginia, 1997; Nkem et al., 2005; Porazinska et al., 2002). The previous studies show that the water content was one of the main factors to drive its distribution and *S. lindsayae* prefers living in the soil with moisture of 2-5% (Mouratov et al., 2001; Mouratov S. et al., 2001; Treonis et al., 2000). However, Gooseff et al. (2003) found there was no significant difference between *S. lindsayae* population in dry soil and them in the soil under snow, which may because of salinity cause osmotic condition changing in the soils (Adams et al., 2014b). *Plectus* nematodes occur in similar types of soils and sediments with higher moisture in Victoria land, usually found in the areas supporting moss and algae (Fig. 1)(Andrassy and Gibson, 2007; Ayres et al., 2007; Wharton and Brown, 1989). And the soil moisture is also a main factor driving *Plectus* distribution. In maritime Antarctica and McMurdo Dry Valleys, *Plectus* nematodes were found more often in moister soils, and *Plectus* was more likely occur in the deepest soil layer under the moss in maritime Antarctica (Courtright et al., 2001; Mouratov et al., 2001). Moreover, compared to *S. lindsayae*, *P. murrayi* has a preference to the soils with higher NH_4-N, NO_3-N, organic C, and organic C/organic N ratios and lower salinity (<100mS cm^{-1}) (Courtright et al., 2001).

Although their habitat preferences are obviously different in Antarctica, both of *S. lindsayae* and *P. murrayi* tolerate freezing and desiccation stresses in the extremely harsh environment of Antarctica, it is valuable to study their adaptations and evolution to reveal the mechanisms that allow them to survive stressful habitats. According to expressed sequence tags in previous studies, it has shown that 80 transcripts are differently expressed in desiccated nematodes to suffer desiccation stress, including the genes related to metabolism related,

environmental information processing and genetic information processing, furthermore genes related to anti-freezing of *P. murrayi* would increase their expression levels in response to desiccation (Adhikari and Adams, 2011; Adhikari et al., 2009, 2010b). However, it is still not fully understood how their functional genes changing to adapt to specific environmental stresses in different habitats, and thus analyzing of transcriptomes of *S. lindsayae* and *P. murrayi* could provide insight into the survival strategies of these organisms with different environmental stresses. Furthermore, comparison of their transcriptomes will provide additional information that could explain their distribution pattern in Antarctica.

Samples

We collected *P. murrayi* and *S. lindsayae* in the Antarctic McMurdo Dry Valleys (MDV), in which *P. murrayi* were sampled from the area near streams in Green Creek, *S. lindsayae* were sampled from many glaciers pound in Taylor Valley. Soil samples were collected up to 10-cm deep using clean plastic scoops and placed in sterile 24-oz Whirlpack bags, then sealed and shipped frozen at -20˚C to Brigham Young University (Freckman and Virginia, 1993). *Plectus murrayi* has been grown in the laboratory at 15°C since 2008, which has been found to be the optimal growing temperature for this species (Adhikari et al., 2010a). To create conditions ideal for *P. murray* growth and reproduction, we inoculated prepared agar-sand plates in incubators, added 30μL of stock *E. coli* OP-50 to phosphorous plates, used a sterilized glass spreader to spread the *E. coli* on the plate, and incubated the plates at 37˚C for 2 days. Then we transferred the nematodes onto the plate and allowed them to stay at 27˚C for 1 week, after being kept at 15˚C in an incubator for 3 weeks before transfer to the prepared plates (de Tomasel et al., 2013). *S. lindsayae* samples were collected from MDV in 2015 and were washed directly out from soil samples with sugar solution centrifugation extraction methods (Knapp-Lawitzke et al., 2014) and

placed by hand into 1.5 ml tubes. Nematodes (of both species) were picked and stored in RNAlater solution and were washed twice with 5% solution of phosphate buffer saline (PBS) before RNA extraction. Total RNA for Quantitative real-time PCR was extracted using NucleoSpin RNA kit (Macherey-Nagel) and RNA library are prepared with KAPA stranded RNA-seq kit. We qualified them on a bioanalysier before the library preparation and sequenced them on illumine Hiseq 2500 in the sequencing center of Brigham Young University.

Geochemistry of the habitats harbored by *Scottnema lindsayae* and *Plectus murrayi*

We collected data from LTER Dry valleys website, and compared the soil geochemistry of Willett Cove and soil from Fryxell basin. According to previous studies, *P. murrayi* found more frequently in the soil of Willett Cove while *S. lindsayae* occurs more in Fryxell basin (Barrett et al., 2006). The abundance of nematodes of *S. lindsayae* and *P. murrayi* was shown in table 1 and the soil geochemistry was shown in figure 2.

We found there was no *S. lindsayae* showed in soils of Fryxell basin and no *P. murrayi* showed in soils of Willet Cove. Moreover, the water content in Willett Cove is significantly higher than it in Fryxell basin ($P<0.001$), the temperatures of these two habitats are similar averagely. The organic C, N, Ammonium contents are higher in soils of Willett Cove than them in Fryxell basin ($P<0.05$), while there is no significant differences on their C : N ratio and Nitrate from soils of Willett Cove and Fryxell basin.

Data filtering

Raw reads of *S. lindsayae* and *P. murrayi* are sequenced by the sequencing center of Brigham Young University and submitted in Genbank (Table 2). For both datasets, FastQC and Trimmomatic (v 0.36) (Bolger et al., 2014) were applied, ILLUMINACLIP: TruSeq3-PE.fa:2:30:10 was used for adapters removing, reads with quality lower than 3 have been

removed by LEADING:3 AND TRAILING:3, 4-base wide sliding window scanned the reads and cutting when the average quality lower than 15, the reads smaller than 36 bp have been dropped.

Transcriptome assembly

We used Trinity (Haas et al., 2013) to assemble the raw reads from both *S. lindsayae* and *P. murrayi*. Single-end default parameter was used with bowtie (V-2.2.5) (Langmead and Salzberg, 2012). Open reading frames were predicted by Transdecoder in Trinity. The transcriptomes of *S. lindsayae* and *P. murrayis* were sorted and prepared for NCBI transcriptome shot gun assembly (TSA) submission, and the NCBI submission information is shown below in Table 2. Blobtools (Laetsch and Blaxter, 2017) were used for contamination screening and low quality scaffolds and contamination sequences were trimmed and excluded. The total number of transcripts from the assemblies of *P. murrayi* and *S. lindsayae* are 31,165 and 29,402 with a mapping rate are 74% and 76% respectively, which indicates the de nova assembly with raw reads dataset quality is acceptable with limited amounts of nematode samples (Table 3).

Annotation

Assembled transcriptomes of *P. murrayi* and *S. lindsayae* were both run through Trinotate pipeline:

Swissport and Pfam databases were built by Trinotate (V 3.1.1). Trinity transcripts were searched for sequence homologies using BLASTX, and RNAMMER was used to identify potential rRNA transcripts. We ran blastx and blastp for these two transcriptomes. HMMER was used for identifying protein domains. Signal peptides were predicted by signal and transmembrane regions were predicted by tmHMM. RNAMMER was originally developed to identify rRNA genes in genomic sequences. To have it identify rRNA sequences among our

large sets of transcriptome sequences, we first concatenated all the transcripts together into a single super-scaffold, ran RNAMMER to identify rRNA homologies, and then transformed the rRNA feature coordinates in the super-scaffold back to the transcriptome reference coordinates. Finally, the annotation report was generated from Trinotate with default settings (Table 4, 5).

Transcriptome Quality and Comparisons

Assembled transcriptome metrics showed an acceptable percentage (over 70%) of reads mapping back to each transcriptome (Table 3) indicating qualified assemblies. TransRate scores ranged from 0.1 to 0.59 for *P. murrayi* and 0.13 to 0.57 for *S. lindsayae*, which were used for quality assessment, and bench-marking universal single-copy orthologs (BUSCO) v. 1.1.1 (Simão et al., 2015; Waterhouse et al., 2017) results using the nematode dataset (downloaded in March 2018) indicated that both transcriptomes have a moderate level of completeness (over 45%). Because of no reference genome or transcriptomes for our assembly, and some novel transcripts have been detected in our assembly, there may be some partial transcripts contained in both datasets, which requires more sequencing from other Antarctic nematodes species to obtain complete transcriptomes.

Cufflinks (V 2.2.1) (Roberts et al., 2012) analysis was run for the comparison between *S. lindsayae* and *P. murrayi* and different gene expression patterns were found (Figure 3). Since *S. lindsayae* and *P. murray* are distributed in significantly different habitats in Antarctica, we aimed to determine differences in their individual functional gene expression patterns and reveal the mechanisms that promote their specific tolerances to abiotic factors in the environment. For example, *S. lindsayae* prefers high salinity and dry soils while *P. murrayi* prefers wet and C, N, P rich soils. Here, we applied TMM normalization to generate a matrix of normalized FPKM values across both datasets. Although the raw fragment counts are used for differential

expression analysis, the normalized FPKM values are used below in examining profiles of expression across different samples, and are shown in heatmaps (Figure 4) and related expression plots.

P. murrayi had 8,296 unique genes, while 8,917 genes were also found in *S. lindsayae*. According to the cufflinks analysis, we selected the top 50 significantly differently expressed genes (fig. 4) (alpha=0.05). sup-26, rrn-3.1 and B0457.6 were up-regulated in *S. lindsayae* and the other 47 genes were down-regulated, we searched the functions of these 50 genes and found some stress related genes up-regulated in *P. murrayi* (table 7). Furthermore, there are some functional genes related to environmental stress tolerance found in both *P. murrayi* and *S. lindsayae*, and no significant differently expressed in these two transcriptomes (table 6).

Discussion

The data we collected from previous study by Barret (2006) and his colleagues showed the soils in Fryxell basin had lower water content, organic C, N and NH_4^+-N than them of soils from Willett Cove, the soils temperature of Fryxell basin and Willett Cove from 5 cm depth are similar averagely. And there was no *S. lindsayae* found in Willett Cove and no *P. murrayi* found in Fryxell basin, which suggests *P. murray* prefer in more productive soils with higher moisture compared to *S. lindsayae* occurs more frequently in the dry and acid soils (Adams et al., 2014a; Barrett et al., 2006). Moreover, the nutrient resources and physical properties make habitats have different biodiversity, and extremely constrains on water and organic matter could also select on the transcriptome, even more strongly. We found some stress tolerance related genes in *P. murrayi* and *S. lindsayae* express similarly in our transcriptomes, which suggests those genes related to adaptation could be conserved in Antarctic nematodes and necessary for them to survive in the dry and cold habitats of Antarctica.

Our comparative transcriptomic analysis shows different expression between the orthologous gene pairs of *P. murrayi* and *S. lindsayae* (figure. 3, 4). The divergence between them indicated that evolutionary constraint acting at genetic expression level. Transthyretin-related proteins (ttr) are a family of proteins related transthyretin that widespread in both invertebrate and vertebrate organisms, these proteins most are involved in numbers of metabolic pathway (Matiollo et al., 2009; Saverwyns et al., 2008). We found TTR-17, 50 in both *P. murrayi* and *S. lindsayae*, but it is higher expressed in *P. murray* compared to *S. lindsayae*, which suggests *P. murryai* might have higher metabolic rate with more resources of nutrients and water. Ubiquitin genes are highly conserved and some studies show it maybe stress-inducible genes (Bond and Schlesinger, 1985; Finley et al., 1987; Graham et al., 1989; Jones et al., 1995). However, Ubq-2 has also been found in all life stages of *C. elegans* and been shown there is no significant expression changing induced by heat stress(Jones et al., 1995). In our study, *S. lindsayae* survive in dryer and more acid soil habitat than *P. murrayi*, and the Ubq-2 expression was found decreased in *S. lindsayae*, which may provide another evidence that Ubq-2 gene were not related to temperature or water stresses. Ribosomal proteins related genes (Rps, Rpl, Snr-1) were expressed more in *P. murrayi* that may because of higher Phosphorus availability according to Growth rate hypothesis (Elser et al., 2003; Sinsabaugh and Follstad Shah, 2010). Skr-3, the skp-1 related ubiquitin ligase complex, involves in cell proliferation, morphogenesis and meiosis (Nayak et al., 2002), which has also found more in *P. murrayi* than in *S. lindsayae* and this may explain their different development strategy in divergent habitats. Sip-1, Stress response gene, is one of heat shock proteins was down regulated in *C. elegans* under toxic condition (Pradhan et al., 2018). Sip-1 was up-regulated in *P. murrayi* to response to harsh abiotic environmental factors more than it in *S. lindsayae*, it may suggest that *S. lindsayae*

adapt to Antarctic limited life supporting soils better than *P. murrayi*. The genes related to development (Mrps-6 and Cpn-3) were up-regulated in *P. murrayi* showed higher metabolic and growth rate due to different habitats properties.

Not like *P. murrayi*, it is difficult to culture *S. lindsayae* on artificial media, and because their divergent distribution in Antarctica, we suggested that their functional genes related to environmental stress tolerance should evolve to adapt to the Antarctic harsh soil habitats. Except hypothetic protein coding genes, the genes associated with 26S ribosomal RNA was up-regulated in *S. lindsayae*, and most of the other ribosomal protein genes were down-regulated. Also, the genes associated with nervous system development were up-regulated in *S. lindsayae*, but other down-regulated genes in *S. lindsayae* are most related to stress-induced proteins, cellular transmission and development metabolisms (table 6). By assembling transcriptome datasets for Antarctic nematodes, more genetic evidence will provide that will increase our understanding of Antarctic nematodes' adaptations and evolution and might establish a model to predict nematode distribution shifts along with climate changes. Furthermore, new transcriptomes of *P. murrayi* and *S. lindsayae* allowed us to studying on the evolution of those functional genes related to stress resistance expression in Antarctic nematodes, which has not been studied completely in previous studies.

Availability of supporting data

The filtered raw reads, assembled and annotated transcriptomes of *P. murrayi* and *S. lindsayae* have been submitted to GenBank as a TSA samples and BioProject numbers shows in Table 1. Datasets supporting the results in this study are available in the GigaScience repository, GigaDB.

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Figures and Tables

Figures

Figure 1. The landscape of different habitats in Antarctica. The left one shows the soil from Hjorth hill with higher moisture and the right one shows the soil from south shore of Lake Hoare that is dry and cold.

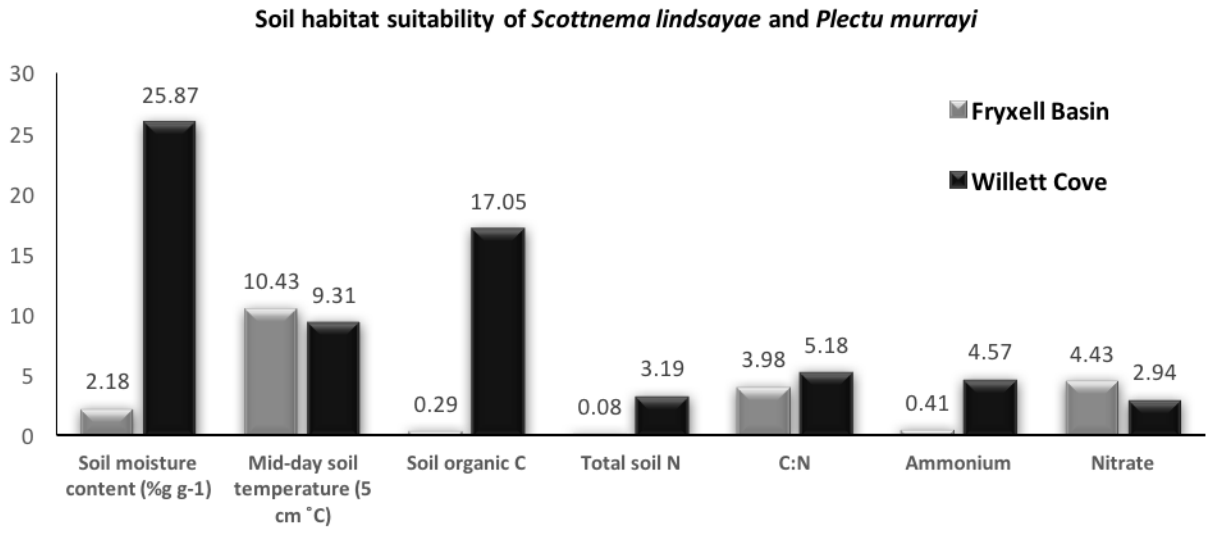

Figure 2. Soil geochemistry and physical properties of the habitats of *Scottnema* and *Plectus*

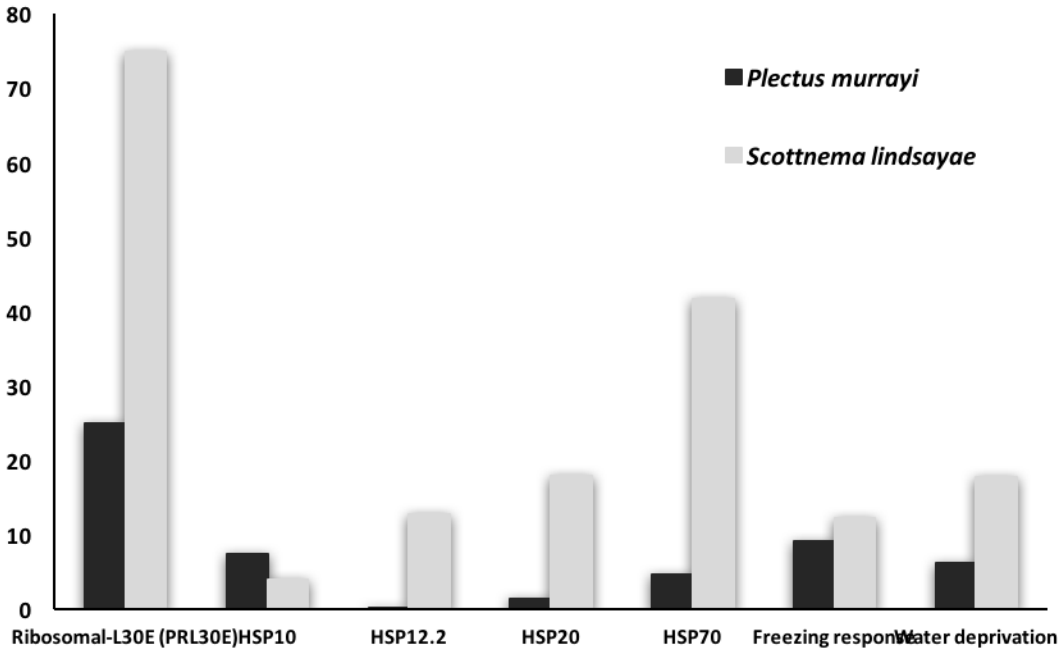

Figure 3. Stress tolerance related genes expression comparison in *P. murray* and *S. lindsayae* (P=0.05).

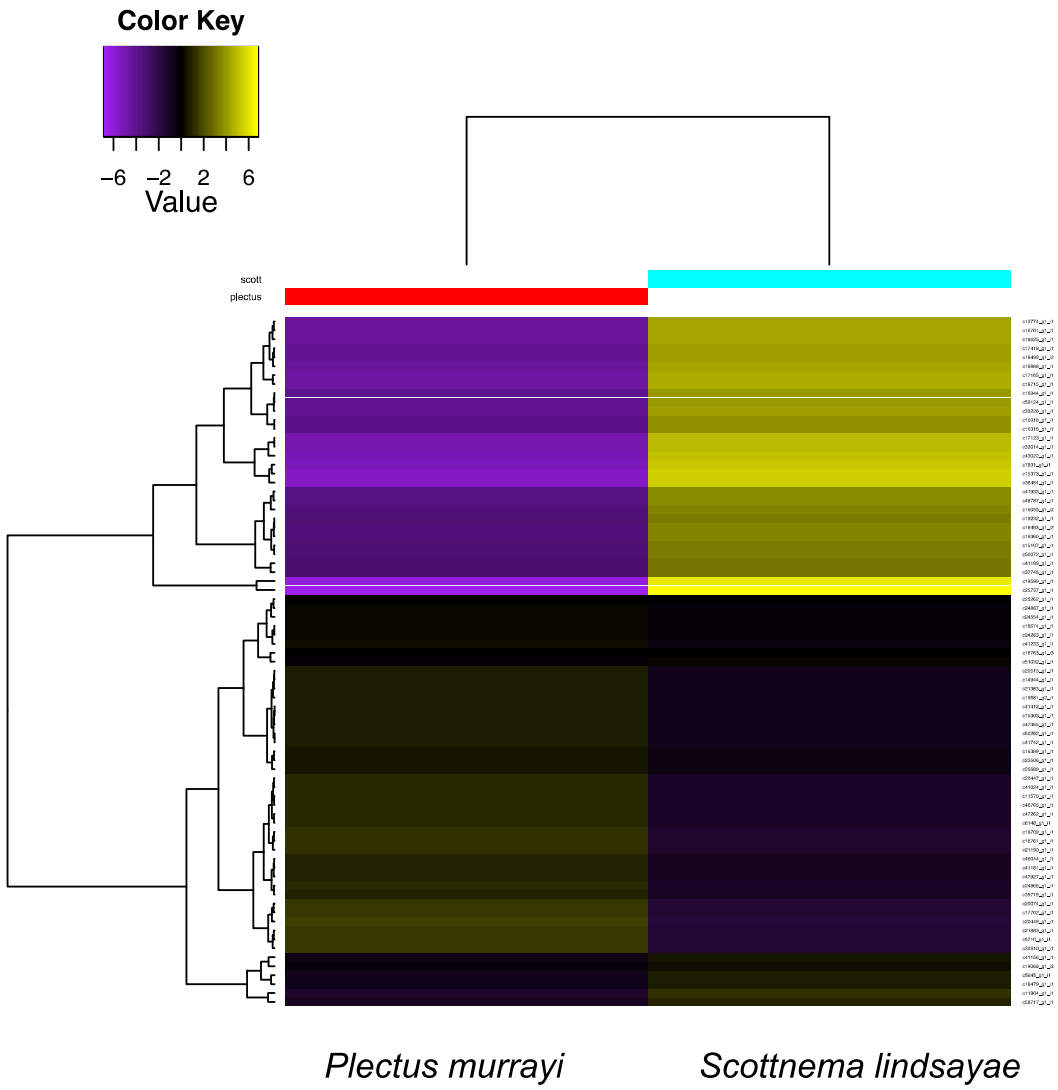

Figure 4. The heatmap with transcripts clustered along the vertical axis and samples clustered along the horizontal axis. The expression values are plotted in log2 space and mean-centered and show upregulated expression as yellow and downregulated expression as purple.

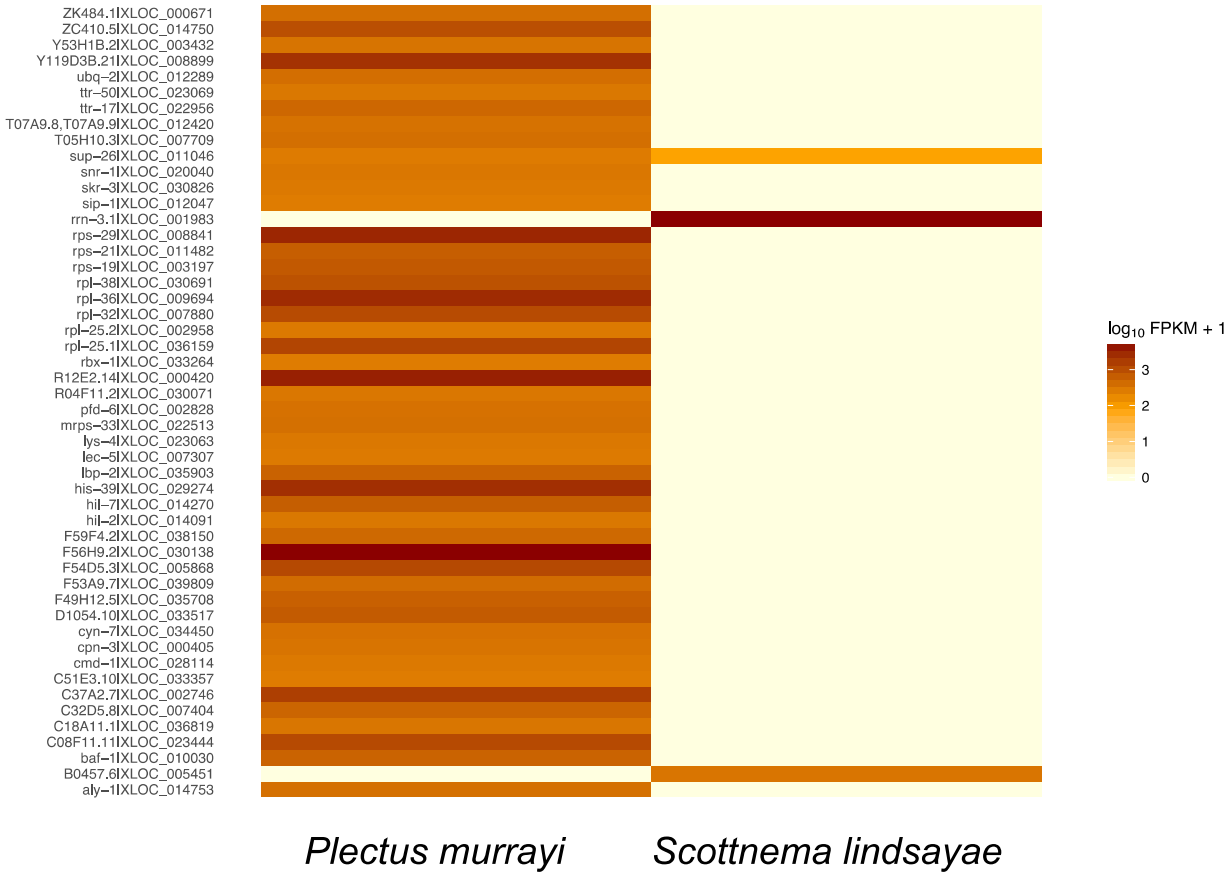

Figure 5. Heatmap of top 50 different expression genes between *P. murrayi* and *S. lindsayae*.

Tables

Table 1. Abundance (Kg soil^{-1}) of *Scottnema* and *Plectus* (Barrett et al., 2006)

Study Site	*Scottnema lindsayae*	*Plectus spp.*
Fryxell Basin	1052±348	0
Willett Cove	0	241±60

Table 2. Accession number of sequences reads and assembled transcripts of *P. murrayi* and *S. lindsayae*

Antarctic nematodes	Reads	Size(Gb)	Short read Archive	BioSample	BioProject
Plectus murrayi	277,590,43	4.6	SRR6827978	SAMN04625768	PRJNA437992
Scottnema lindsayae	205,706,08	3.3	SRR6827990	SAMN08688980	PRJNA437994

Table 3. Transcriptome assembly statistics of *P. murrayi* and *S. lindsayae*.

	Plectus murrayi	*Scottnema lindsayae*
Total number of transcripts	31165	29402
Average transcript length	449.49	538.41
Total assembled bases	15566609	15830200
N50	597	674
%GC	52.88	42.04
%mapping	74.11	76.91

Table 4. Annotation reports of *P. murrayi* and S. *lindsayae*.

	P. murray	*S. lindsayae*
Gene_id	31,165	29,402
Transcrip_id	31,165	29,402
Transcript	31,165	29,402
Sprot_top_Blastx_hit	14,913	21,925
Gene ontology Blast	14,799	16,868
Prot_id	14,680	16,868
Prot_coords	14,680	16,868
Peptide	14,680	14,065
Kegg	13,507	13,832
Eggnog	13,269	12,648
Sprot_top_blastp_hit	10,226	12,521
Pfam	8,722	10,485
RNAMMER	5,742	9,470
Gene_ontology_pfam	5,489	6,017
TmHMM	1,617	2,159
Signal P	564	703

Table 5. Annotation summary from Pfam of *P. murrayi* and *S. lindsaysae*

	Plectus murrayi	*Scottnema lindsayae*
Number of proteins with PFAM domains identified	15,586	16,131
Number of proteins with Gene Ontology Terms	12,331	13,236
Number of proteins	13,038	14,050
Number of transcripts	13,038	14,050

Table 6. Up-regulated genes and functions in *P. murrayi*

Gene name	Function	
Ttr-17, 50	TransThyretin-related family	
Ubq-2	ubiquitin	DNA replication, Cytoplasmic ribosomal protein
Rps	ribosomal protein small subunit	Ribosome biosystem
Rpl	ribosomal protein large subunit	Ribosome biosystem
Snr-1	small nuclear ribonucleoprotein sm D3	RNA-binding, processing, splicing
Skr-3	skp1 related ubiquitin ligase complex	Ligase activity
Sip-1	stress-induced protein 1	Unfolded protein binding
Rbx-1	ring-box 1	Metal ion binding, zinc ion binding
Pfd-6	probable prefoldin subunit 6	Chaperone binding, locomotion, reproduction
Mrps-6	mitochondrial ribosomal protein, small	Nematode larval development, rRNA binding
Lys-4	lysozyme	Lysozyme activity
Lec-5	galectin	Carbohydrate binding, membrane raft
Lbp-2	fatty acid-binding protein homolog 2	Lipid binding, transporter activity
His-39	histone	DNA binding, locomotion, embryo development
Hil-2, 7	histone H1.2	DNA binding, nucleosome assembly

Cyn-7	peptidyl-prelyl cis-trans	Isomerase activity, peptidyl-prolyl cis trans isomerase
Cpn-3	calponin	embryo development ending in egg hatching
cmd-1	calmodulin	calcium ion binding, metal ion binding
Baf-1	barrier-to-autointegration factor 1	DNA binding, DNA repair
Aly-1	ref/aly RNA export adaptor family	Nucleotide binding, locomotion

Chapter 5

Life history and transcriptomic comparison of *Plectus murrayi* cultured in different temperature and phosphorus

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Abstract

Antarctic McMurdo Dry Valleys are the driest and coldest desert, its extremely low biodiversity and insufficient nutrients availability, make it an excellent model ecosystem to study the relationship between the organism's adaptation and environmental stresses. *Plectus murrayi*, one of Antarctica endemic nematodes that could be cultured in an artificial media. We measured life history traits and reproduction of *P. murrayi* from P-rich and P-poor conditions. And both transcriptomes of *P. murrayi* cultured in P-rich and P-poor were also sequenced to find out differently expressed genes that are related to nematodes development and stress tolerance. We found the available P from the environment could influence the growth rate and reproduction of *P. murrayi*. The nematodes are growing faster and have smaller adult body size when there is more P, while those in P-poor condition have more extended development period but can reach bigger body size at maturity. According to transcriptomic analysis, 21,226 and 30,015 unigenes were found in *P. murrayi* in P-poor and P-rich conditions, of which 10,475 and 14,907 (48.18% and 49.67%) genes found in the database. We found 31 genes were up-regulated and 19 genes down-regulated of *P. murrayi* from P-poor to P-rich conditions from edgeR analysis (top 50), the

GO analysis showed a large number of biological processes were significantly modified between P-poor and P-rich treated transcriptomes. This study will underscore the important relationship between the evolution of life history traits and transcriptome expression pattern.

Introduction

The Antarctic McMurdo Dry Valleys is a polar desert, the annual temperature there range from -17 to -20°C averagely, and annual precipitation averages less than 10 cm water equivalent as snow (Doran et al., 2002; Fountain et al., 2014). The landscape of the dry valleys is a desert that composed discretely of glaciers, arid mineral soils, streams from melted water and perennially ice-covered meromictic lakes (Priscu, 1998). The contemporary structure and function of dry valley ecosystem are shaped by the geochemical legacies of past environments and geological events. In the dry valleys, nematodes are the most widely distributed invertebrates across various types of habitats (Treonis et al., 2000). The structure and geographic distribution of Antarctic Dry Valley nematode are primarily driven by water and some abiotic factors, especially organic matter and salt concentration (Barrett et al., 2007; Bate et al., 2008; Freckman and Virginia, 1997; Powers et al., 1998).

In the genus *Plectus,* there are four species have been recorded from Antarctica: *P. murrayi*, *P. antarcticus*, *P. frigophilus*, and *P. acuminatus*(Adams et al., 2014; Adams et al., 2006; Andrassy, 1998), among them, *P. murrayi* and *P. frigophilus* are only found in Antarctica (Adams et al., 2014). *P. murrayi* prefers to occur in higher moisture soil habitats with relative higher NH_4-N, NO_3-N, organic C and lower salinity compare to other Antarctic nematodes (Courtright et al., 2001). Compare to *Scottnema* nematodes, *P. murray* are more frequently found near the snowmelt patches from Antarctic Dry Valleys, it likely distributed driven by the water contents in the soil (Adams et al., 2014; Adams et al., 2006). *P. murrayi* is highly specialized and adapts to this extreme dry and cold desert, and it is one of the Antarctic nematodes that can be cultured on artificial media (Adhikari et al., 2010a; de Tomasel et al., 2013), and has an important and unique phylogenetic position relative to an origin of the Secernentean radiation.

Furthermore, its high tolerance to extreme environmental stresses (desiccation, freezing, high concentration of heavy metal, etc.) has also been studied by many researchers (Adhikari et al., 2009; Hendriksen, 1983; Nkem et al., 2005). Thus, *P. murrayi* is emerging as a new model nematode to study on evolutionary adaptation response to poor life-supporting environment (Blaxter et al., 1998; Holterman et al., 2006). The previous studies show it will take *P. murrayi* a year to finish the life cycle (Yeates et al., 2009) in the wild and around 6-8 weeks in the laboratory at 15 ˚C on artificial media (de Tomasel et al., 2013). To survive in Antarctic extremely acrid soil habitats, *P. murrayi* evolves adaption strategies that allow it tolerant desiccation and freezing with short growing season (Treonis et al., 2000; Wharton et al., 2005; Wharton and Raymond, 2015), Adhikari and his colleagues (2010b) found exposed them to slow desiccation and freezing would result in metabolism and signal transduction-related genes expression differently with nematodes exposed to fast desiccation and freezing. Some studies also investigated the genetic response from *P. murrayi* that suffered in anhydrobiotic condition (Adhikari et al., 2009; Hendriksen, 1983; Sandhove et al., 2016). Moreover, gene expression studies showed that pre-exposure to light stress improve survival through stronger stress, for example, the Antarctic nematodes that exposure to slow dehydration promotes their extreme desiccation survival and improve cold tolerance (Adhikari et al., 2010b; Wharton and Raymond, 2015).

The link between cellular P content and organismal growth is explained by the Growth Rate Hypothesis (GRH), suggesting that high organismal P-content reflects increased allocation to P-rich ribosomal RNA needed to meet the protein synthesis demands of increased growth rates (Elser et al., 2003; Elser and Hamilton, 2007). Phosphorus performs critical functions in number of biological processing, which is significantly limited in Antarctic soils (Blecker et al., 2006;

Velasco-Castrillón et al., 2014), studies on the development of *P. murrayi* related to Phosphorus (P) constrains in Antarctica is little but important, and phosphorus plays a key role on ribosome building, it is valuable to study it cross with the development of organisms, particularly in P extremely limited environment. Differences in P-content observed between the Ross Sea and Taylor II tills may be explained by the differences in situ physical, chemical and biological processes (Blecker et al., 2006). Previous work has demonstrated that soils collected from Ross Sea tills had to bother higher total P and soluble phosphate content (Bate et al., 2008; Blecker et al., 2006). By sequencing the transcriptomes that *P. murrayi* growing in different P concentrations, we tried to find evidence that insight into the regulation of stressed induced transcripts during different P concentrations.

Sequencing the transcriptome makes us obtain the complete expressed RNA transcripts of a cell, a tissue, or a whole organism (Trapnell et al., 2010). It is an excellent method to understanding the cellular activities in different stages or different conditions. RNA sequencing is also a useful and informative tool for studying on the organism without genome sequences (Bruno et al., 2010; Cheng et al., 2015; He et al., 2015; Król et al., 2016). We used this technology to understand the Antarctic nematodes *P. murrayi* growing in different P conditions and their transcriptomes are firstly sequenced from Antarctica that could establish a RNA transcripts database for future investigations of other Antarctic nematodes.

By studying on *P. murrayi*, we hope to be able to reveal the new connections between adaptation and stoichiometric constraints (including the GRH and genomic streamlining), to find out these functional genes expression patterns that are responsible for stress tolerance. Based on studies of short term and long term responses of *P. murrayi* to environmental stresses at transcriptomic level, it is possible to reveal the relationship between genome evolution and

ecosystem complexity, as well as the role of elemental stoichiometry in shaping communities under phosphorus limited environment.

Here in this paper, we cultured *P. murrayi* in poor and rich P media since 2008, and observed its life history to measure how P constrains impact on its development and body sizes. We also systematically compare the transcriptomes of *P. murrayi* cultured in different P conditions at 15 ˚C. The transcriptome datasets were used for transcriptome analysis and were aimed to study the different expressed genes when *P. murrayi* cultured in different P conditions. Among differently expressed genes, we aim to find the gene family associated with development, stress tolerance and P cycling, to explain its adaptation strategy of survival in the dries and coldest desert on the earth and tolerance of extreme poor-P content with few primary producers.

Materials and Methods

We collected *P. murrayi* from Antarctica in season 2008-2009. The samples were taken by clean plastic scoops from the soil up to 10-cm depth, and placed in sterile 24-oz Whirlpack bags, sealed and shipped frozen at -20˚C to Brigham Young University. *P. murrayi* were cultured in P-poor and P-rich media at 15 ˚C, which has been found to be the optimal growing temperature (Adhikari et al., 2010a; de Tomasel et al., 2013). We picked and transferred 20 pregnant females to fresh media individually that prepared with OP-50 *Escherichia coli* on. All plates have been observed every day and counted the number of eggs they laid, body sizes of *P. murrayi* from each group have been measured by CKX 41 Olympus inverted microscope.

P. murrayi has been maintained in P-rich and P- poor media for at least 48 months. As we mentioned above, it takes 6-8 weeks for *P. murrayi* to finish an egg to egg life cycle. We inoculated prepared rich/poor phosphorous agar plates, added 30μL of stock OP-50 to phosphorous plates, used a cotton swab to spread the bacteria on the plate and incubated them at

37˚C for 2 days. Then we transferred nematodes onto the plate and bred them at 27˚C for 1 week, followed by incubation at 15˚C for 3 weeks before repeating the process for the next (Adhikari et al., 2010a). We used phosphorous sand agar media for growing nematodes, which includes 15g Agar, 965 mL H_2O, 20 ml BMB for both P-poor and P-rich plates, and 1.033 mg K_2HPO_4 for P-poor and 10.33 mg K_2HPO_4 for P-rich . The pH was adjusted 7.0, H_2O was added to 1.0 L, and the mixture was autoclaved for 20 minutes at 120 ˚C. Sand was poured on cooled plates, which were then stored in 4 ˚C.

2000 *P. murryai* adults from P-rich and P-poor conditions were picked by hand and stored in RNAlater at 4 ˚C for RNA extraction. Total RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel) and qualified by Bioanalysier, then the library preparation with KAPA standed RNA-seq kit and sequencing applied on illumine Hiseq 2500 in sequencing center of Brigham Young University. The RNA-seq datasets of each group of *P. murryi* were qualified through FastQC and trimmed with Trimmomatic (v 0.36) (Bolger et al., 2014). We used Trinity to assemble the raw reads from *P. murrayi*. Single-end default parameter was used through with bowtie (V 2.2.5) (Langmead and Salzberg, 2012). Open reading frames were predicted by Transdecoder (Haas et al., 2013). Total annotation was reported from Trinotate (V-3.1.1) with outcomes from Trinity (Haas et al., 2013), HMMER (Finn et al., 2011), PFAM (Punta et al., 2011), tmHMM (Krogh et al., 2001), KEGG (Kanehisa et al., 2012), GO (Ashburner et al., 2000), eggnog (Powell et al., 2012), RNAMMER (Lagesen et al., 2007). Blobtools (Laetsch and Blaxter, 2017) was used for contamination screening and low quality scaffolds and contamination sequences were trimmed and excluded.

Results

We measured the total number of offspring of *P. murrayi* under P-rich and P-poor conditions, and track their reproduction rate and period respectively. We found *P. murryai* in P-poor condition have longer reproduction periods and slower reproduction rate with similar total number of eggs (Figure 1). Moreover, we found that *P. murrayi* reached the reproduction peak per day earlier in P-rich media compare to those in P-poor condition.

We found there is no significant difference in the total number of offspring between *P. murrayi* cultured in P-rich and P-poor conditions (Paired t-test, $P>0.05$). Moreover, the average total number of eggs was 19.33, 19.80 for *P. murrayi* in P-rich and P-poor conditions respectively (Figure 1). The reproduction rates of *P. murrayi* reared in P-rich and P-poor media are not significantly different ($P\geq 0.05$). Moreover, the *P. murrayi* reared in P-rich condition reproduce shorter time than them in P-poor.

Although there is no significant difference in the total number of eggs between *P. murrayi* reared in P-rich and P-poor media, we found the nematodes reared in P-poor media produced longer time than those raised in P-rich media. Also, we measured their egg volumes and hatch rates for nematode from P-rich and P-poor media. The average egg size of *P. murrayi* is 1.59×10^{-5} mm^3 and 1.08×10^{-5} mm^3 respectively. The average hatch rates of *P. murrayi* reared in P-rich and P-poor media was 70.93% and 57.30% respectively.

The adult body volumes of *P. murrayi* reared in P-rich were significantly smaller than those reared in P-poor conditions (t-test, $P<0.05$), while body length displayed the opposite trend (t-test, $P<0.05$). Moreover, the nematodes required longer growth times to reach reproductive maturity in P-poor conditions than when grown in P-rich condition.

We also measured the developmental period of *P. murrayi* cultured in P-rich and P-poor conditions. And there is a significant difference between some life stages of development of *P. murrayi*, but not every stage (Figure 2). In *P. murrayi*, the time from egg to young adult, to adult and egg laying are different. It took less time for *P. murrayi* growing in P-rich media to reach those stages than those grown in P-poor media.

The transcripts assembler by Tophat and Trinity with Bowtie2, and mapping rate of *P. murrayi* from P-rich and P-poor are 94.4% and 91.1% (Table 1). According to BUSCO, both of the assembler is moderate complete. The N50 value peak shows over 80% of the expression data, which suggests high quality transcriptome assembly (Figure 3).

Comparison of *P. murrayi* transcriptomes (Figure 4) from P-rich and P-poor condition revealed genes coding for collagen, hexose-binding lectin, CBR-TAG-297 protein, C-type LECtin F-box B protein, Galactosylxylosyl protein 3-beta-glucuronosyl transferase, Lipase-related proteins and O-Acyl transferase homologs were up-regulated when P is poor. Genes related to Hydroxy-Acyl-CoA Dehydrogenase and Downstream Of DAF-16 proteins were down-regulated in P-poor condition. Therefore, we observed different patterns of gene expression between nematodes reared in same P condition, but different temperatures.

According to our edgeR function genes richness analysis, we located top 50 significantly differently expressed genes in *P. murrayi* in P-rich and P-poor conditions and searched their functions, there are some genes related to larval development up-regulated when there is more available P, and genes related to stressed up-regulated in P-poor condition (Table 2 and 3).

Discussion

In this study, stoichiometric constraints have been shown to be correlated with life history traits and organisms cultured in the lab should possess the same traits as those found in wild P-

poor environments, i.e. delayed maturity and larger adult body size/volume. Our results show the phosphate concentration in different habitats could influence on the population developmental processes of *P. murrayi*. Additionally, *P. murrayi* cultured with more P achieved their peak fecundity earlier than the ones with less P, which might imply that more available P resources to build their proteins, more energy to reproduce, and faster reproduce comes along. Under the same temperature (15 ˚C), nematodes cultured in P-rich environment possess a higher volume of adult body size and shorter of adult body length compare to those cultured in P-poor environment. This could be an adaptive strategy of development that nematodes utilize limited available P from the environment, its response to stoichiometric constraints by reallocating resources and energy in a way that they can grow and reproduce in a limited P content. We did find some changes on life history traits and reproduction patterns from *P. murrayi* in P-rich from P-poor media, which are consistent with the performance of *Scottnema* and *Plectus* found in Antarctic Dry Valleys (see Chapater 2, unpublished data).

P. murrayi live in P-poor have longer reproduction period than those reared in P-rich condition, while the total number of eggs reproduced by both of the groups is not significantly different. Accordingly, we suggest P resources could impact on *P. murrayi* reproduction by slowing their development when there is not enough P, when there is enough P, *P. murrayi* will reproduce at the higher daily rate within shorter entire reproduction period, then reach the similar number of total offspring come from *P. murrayi* with poor P condition. Previous studies show that changes in growing temperature could lead to altered life history traits on nematodes, and under different environmental stresses the growth rate and development could respond (Ball and Baker, 1996; Lind et al., 2017; Lind and Johansson, 2011). Moreover, nutrient availability can drive microbial community structure and composition, which is important when considering

development and reproduction in nematodes(Shapira, 2017). In our experiment, the temperature was 15 ˚C for culturing and experiments, which reflects the ambient soil temperature of the MCM dry valleys for the two locations with different P content is similar. A number of researchers consider that life span will decrease when the organisms invest more resources into reproduction (Boonekamp et al., 2015; Flatt, 2011), thus our study provides an experimental fact to support this point. Development and duration of juvenile growth are responsible for the differences in life span. Lind and his colleagues (Lind et al., 2017) suggested that development showed a strong response to stress selection and had a significant effect on longevity. In our study, the life span of *P. murrayi* in different P conditions is similar. Moreover, since the growth rate of *P. murrayi* in P-poor is slower than in P-rich , it shows that P availability plays an essential role in their development and growth rate, and since *P. murrayi* is endemic to Antarctica, it could be a model organism to study on this adaption strategy survive in this available P extremely limited habitat.

Current work involves revealing how the environmental stresses impact genome architecture and gene expression. In our study, transcriptomic analysis of *P. murrayi* under different P concentrations can provide evident that insight to the timing and expression of functional genes associated with environmental stress tolerance. By sequencing transcriptomes of *P. murrayi* in different available P-rich and P-poor growing conditions, we tried to understand how these environmental stresses impact on *P. murrayi* at the genetic level and how these functional genes related to its life history and development. We showed that expression of genes related to available environmental phosphorus uptake is differentially expressed under P-poor and P-rich conditions. Some studies showed animals with P-poor diet growing lower and have a

lower protein efficiency ratio. Thus deficient P availability would reduce protein efficiency ratio and suggests P utilization is a key role in the development (Lu et al., 2017).

A total of 21,226 and 30,015 unigenes were generated for *P. murrayi* in P-poor and P-rich , of which 10,475 and 14,907 (48.18% and 49.67%) unigenes were annotated against the NR, Swiss-Prot, KEGG databases using the BLASTx and BLASTn algorithm. And there are some of the genes were not found in any of the databases. These results provide a good foundation for further studies aimed at identifying new genes in *P. murrayi*. Furthermore, of the 21,742 and 31,165 transcripts were mapped to the Nematoda database. These results indicate a high level of gene conservation between *P. murrayi* and *C. elegans*. To understand the mechanisms involved in the reduced growth rate and protein efficiency induced by P-deficiency, the transcriptome of *P. murrayi* growing in P-poor and P-rich cultures were sequenced and analyzed. In total, 31 genes were up-regulated and 19 genes down-regulated between *P. murrayi* from P-poor to P-rich conditions, the GO analysis showed that a large number of biological processes were significantly modified between P-deficient and P-sufficient treated transcriptomes. Most of the differentially expressed genes were involved in the metabolism of carbohydrates, proteins and lipids. The results indicated that many biological processes were significantly influenced by the environmental available P.

To identify the functions of the genes, KEGG pathway analysis was used on differentially expressed genes from *P. murrayi* in P-rich and P-poor conditions. Pathway enrichment analyses show that the cellular process and metabolism was altered in P-rich condition compare to those in P-poor condition. We found some genes related to cell dividing and cycling are up-regulated in P-rich conditions; this may imply the limited P could decrease the cell growing and dividing process, which will increase slower the growth rate in *P. murrayi*. Furthermore, the genes related

to cell decomposition are up-regulated in P poor condition, which suggests the organism could use available P more efficiently when there is limited P in the environment. There are some studies found that carp fed insufficient P exhibited an increased amount of nitrogen excretion. Moreover, P also plays an important role in carbohydrate metabolism (Liang et al., 2012; Lu et al., 2017). Furthermore, genes related to cell development and metabolisms are up-regulated in P-rich condition. This result could explain the *P. murrayi* in P-rich condition grow faster and reproduce at a faster rate. Genes involving in insulin signaling and fatty acid metabolism are up-regulated when P is limited, which suggests P constrains will increase the energy relocation of the nematode. Liu et al. (2013) found that elongation of fatty acids family was very important in long-chain fatty acid biosynthesis.

By studying on the transcriptome comparison, we hope to be able to reveal its strategy to survival in acid cold soil from Antarctica. By understanding the tolerance or resistance to harsh environmental biotic/abiotic stresses of soil organisms, it is possible to discover new strategies about integrity pest management agriculture. Furthermore, the interactions between low diversity soil biota and ecosystem function described in the MDV will lay a foundation for understanding other more complex ecosystem in the world.

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Figures and Tables

Figures

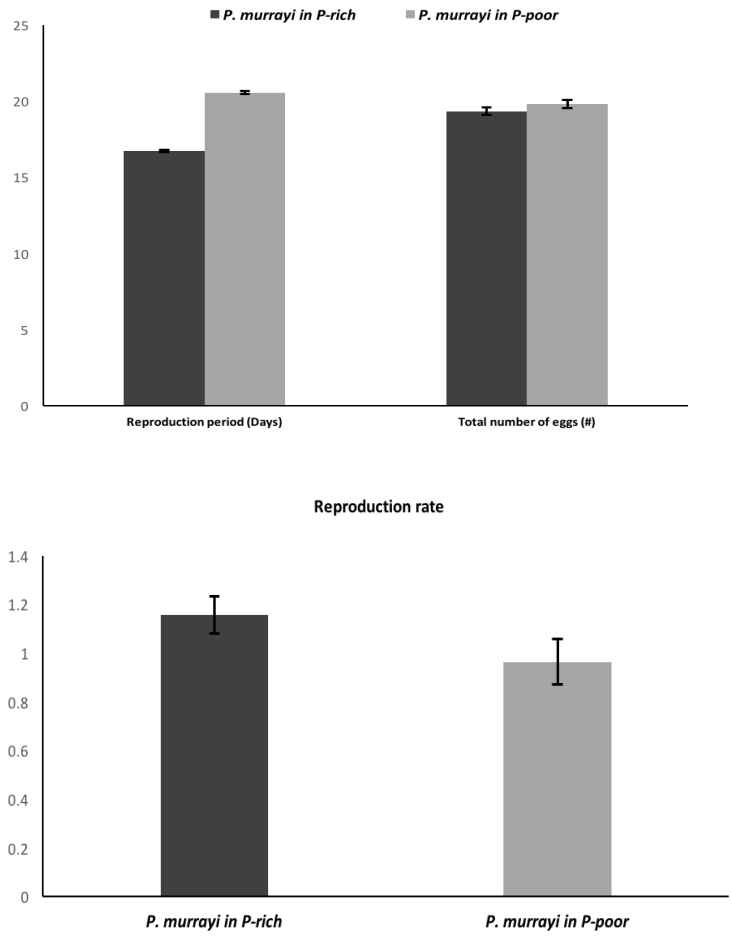

Figure 1. Reproduction and total number of eggs of *P. murrayi* under P-poor and P-rich media. There was no consistent pattern of growth rate and fecundity.

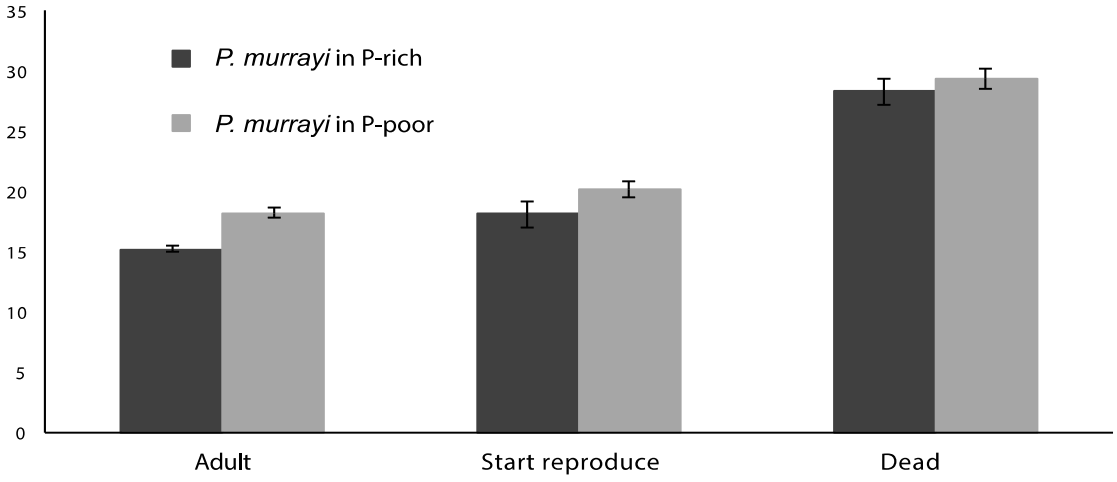

Figure 2. Comparison of the life history stages in *P. murrayi* reared in P-rich and P-poor media. Time to and start of egg laying dates is significantly different at $P \leq 0.05$.

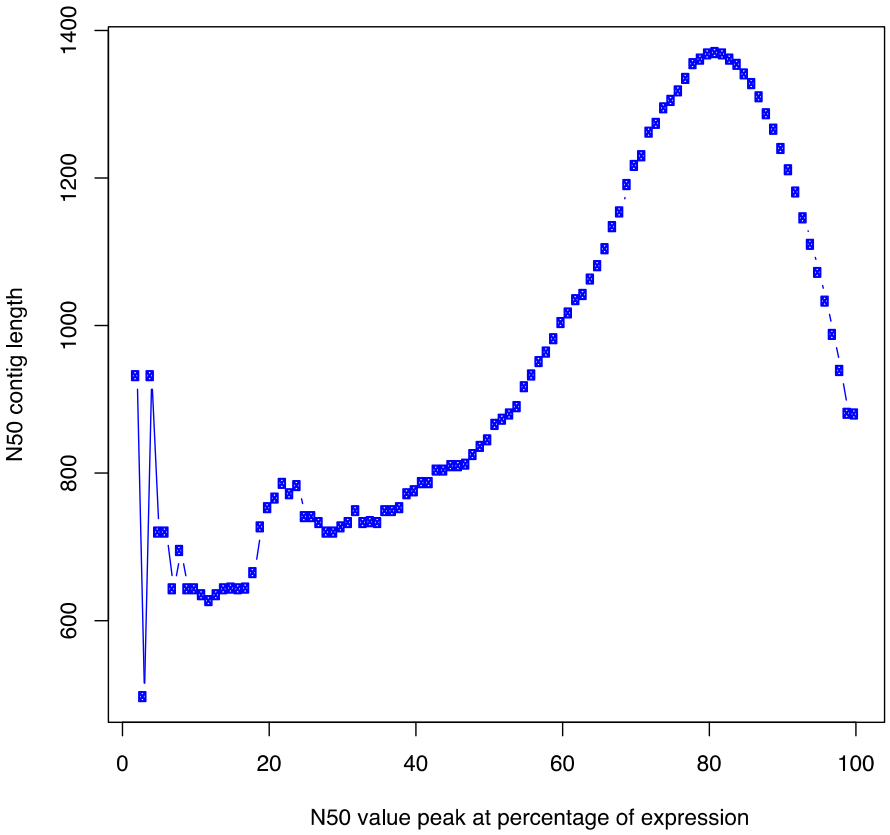

Figure 3. N50 value distribution of transcriptome assembly.

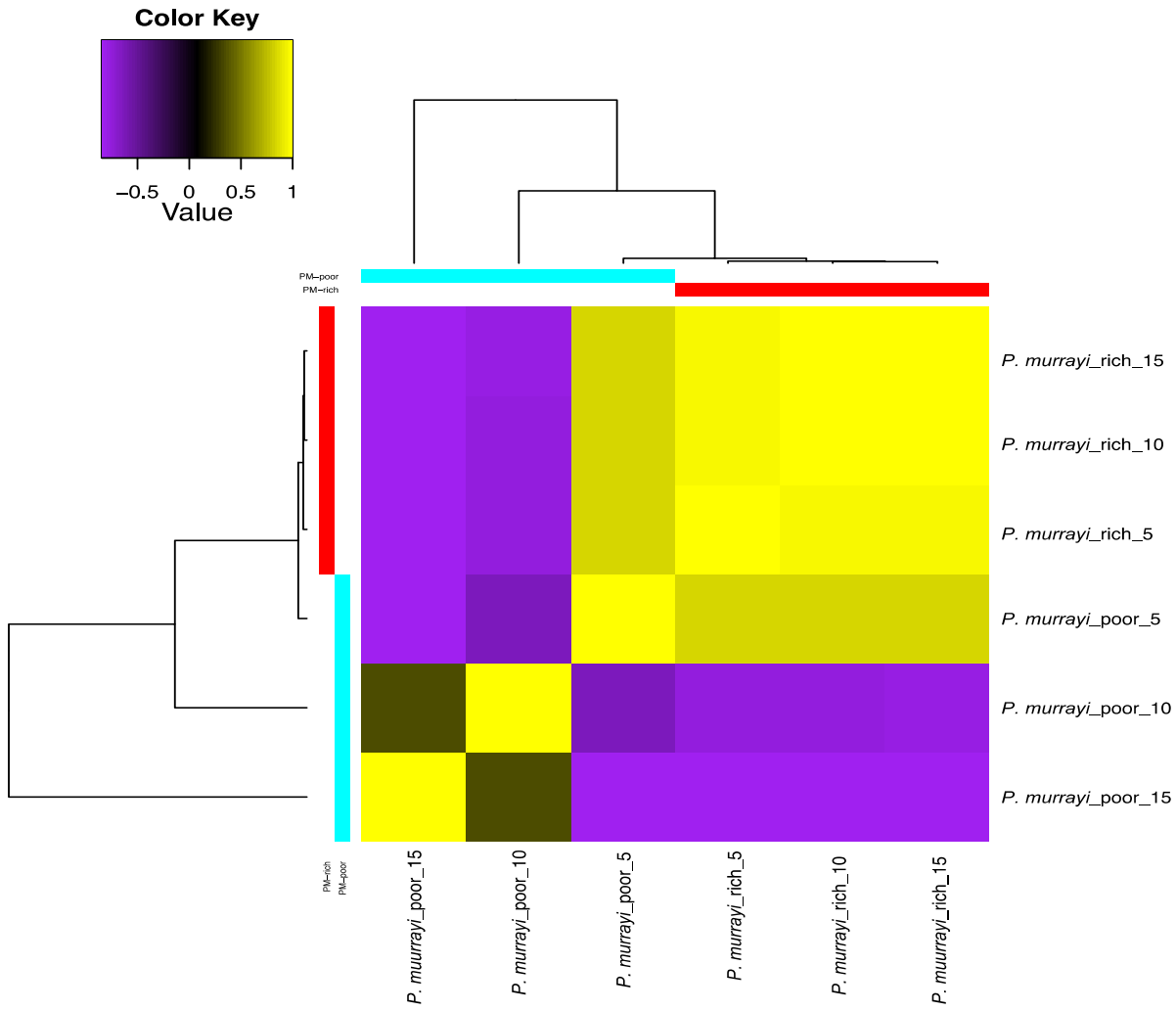

Figure 4. Correlation among different groups, which are *P. murrayi* cultured at 5 ˚C, 10 ˚C, 15 ˚C with P-rich and P-poor ($P=1e^{-3}$).

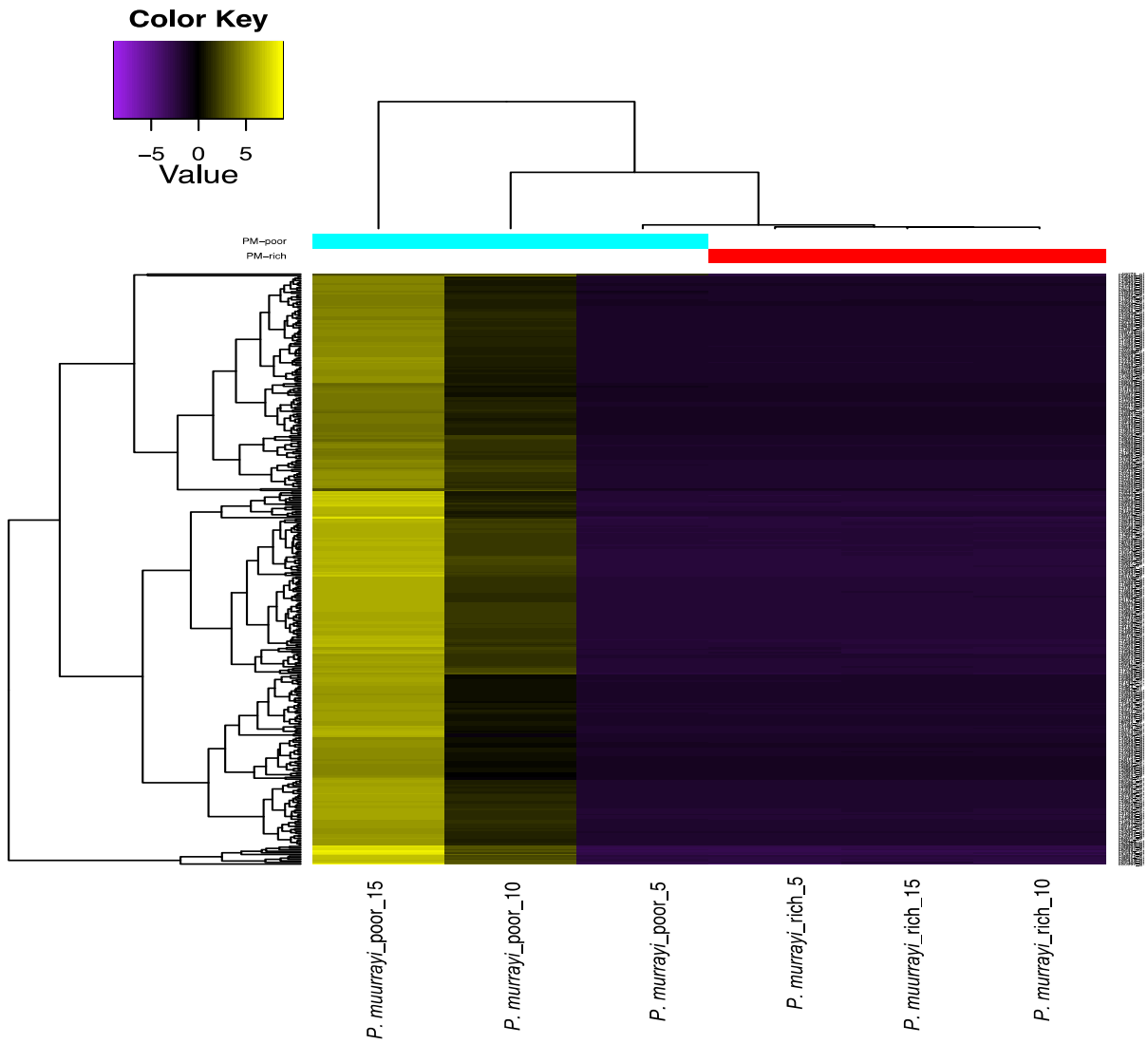

Figure 5. The expression patterns of different groups. The expression values are plotted in log2 space and mean-centered (mean expression value for each feature is subtracted from each of its expression values in that row), and shows upregulated expression as yellow and downregulated expression as purple in *P. murrayi* in P-rich and P-poor conditions.

Tables

Table 1. Transcriptome assembled information.

Sample Name	Input reads	Mapped reads	Multiple alignments	Mapping rate
P. murrayi P-rich	14082412	13287664	6.2% (830331)	94.4%
P. murrayi P-poor	25052710	22831443	6.2% (1406472)	91.1%

Table 2-A. Briefly report the genes of *Plectus murrayi* at 15 ˚C are up-regulated in P-rich

Gene name	Functions
Spp-3,15	Saposin-like protein family
Msd-4	Major sperm protein domain
Mir-22	microRNA 22
His-37	Heat shock protein 37
Fipr-21	Fungus-induced protein related
Cyn-7	Peptidyl-prolyl cis-trans isomerase 7
Cpg-8	Chondroitin proteoglycan 8

Table 2-B. Genes of *Plectus murrayi* at 15 ˚C are down-regulated in P-rich

Gene name	Functions
Taf-13	TBP-associated transcription factor
Sup-26	Suppressor
Ins-6	Probable insulin-like peptide beta-type 5
Hil-3	Histone H1.3
Fip-5	Fungus-induced protein
Far-1	Fatty acid/retinol binding protein
Cnc-2	CaeNaCin
Clec-221	C-type Lectin

Table 3. Functions of genes up-regulated (A) and down-regulated (B) in P-rich at 15 ˚C

Up-regulated functions
G-protein coupled receptor binding, enzyme activator activity
Cell body building
Cell cycle, cellular senescence, chromatin modifying enzymes
Isomerase activity, peptidyl-prolyl cis-trans isomerase activity
reproduction

Down-regulated functions (B)
Eukaryotic transcription initiation, RNA polymerase II pre-transcription events, basal transcription factors
mRNA3'-UTR binding, nucleic acid binding, protein binding
Insulin receptor binding, hormone activity
DNA binding, nucleosome assembly
Lipid binding, body morphogenesis
Molecular function
Carbohydrate binding