Doctoral Thesis

Ecological patterns in soil nematode diversity and community composition along two environmental gradients

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School of Biological Sciences

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Ecological patterns in soil nematode diversity and community composition along two environmental gradients

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ABSTRACT

Nematodes are considered to be the most abundant animals on Earth, playing a significant role in nearly all the world's ecosystems. Because of their key position as primary and intermediate consumers in soil food webs, assessing the possibility of changes in diversity of nematode community structure is seen of great importance.

Trends in diversity and community structure in relation to elevational and time successional gradients have been well documented among a wide range of forms of life, including animals, plants, insects or some larger invertebrates. In recent years, the elevational trends in community structure and diversity of microorganisms have also been discovered, due to advances in extraction and sequencing of environmental DNA. However, so far there has been very little concerted effort to determine how diversity patterns and community structure of soil nematodes vary with these gradients, despite their abundance and important roles in ecosystem processes.

The lag in understanding of nematode communities is partially caused by the lack of an efficient way to identify them. Until recently, all ecological studies of nematodes relied on morphological criteria. However, in the past several years, it has become possible to assess biodiversity of soil nematode by bulk physical isolation of the organisms from soil and extraction of their DNA *en masse*. Massive parallel sequencing of selected marker genes allows taxonomic classification and estimation of diversity and relative abundances. Not only have these molecular-based methods greatly facilitated rapid sampling, they have also revealed a much greater 'hidden' nematode

diversity than was suspected from morphological studies.

Here, the aim was to set out to conduct a more systematic and representative study focusing on soil nematodes, in relation to two environmental gradients. I found diversity of soil nematode communities will differ along these gradients, showing continuously changing patterns, and total nitrogen concentration in soil is a good predictor of community diversity for these patterns in both elevational gradient and primary successional gradient. Moreover, along these gradients, community structure of soil nematodes differed and produced a clear succession of communities.

Keywords: soil nematode, elevational gradient, primary successional gradient, communities, community diversity, community structure, high-throughput sequencing, 18S rRNA gene

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ABBREVIATIONS

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

NGS: Next generation sequencing

SRA: Short read archive

rRNA: Ribosomal ribonucleic acid

NMDS: Non-metric multidimensional scaling

BLAST: Basic local alignment search tool

PCoA: Principal coordinate analysis

RDP: Ribosomal database project

DNA: Deoxyribonucleic acid

MNTD: Mean nearest taxon distance

ses: Standardized effect size

AOA: Ammonia-oxidizing archaea

PERMANOVA: Permutational multivariate analysis of variance

RDA: Redundancy analysis

masl: meters above sea level

RDP: Ribosomal database project

RDA: Redundancy analysis

RMSE: root mean square error

ANOVA: Analysis of variance

MAT:Mean annual temperature

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CHAPTER 1
Soil nematode ecology and the techniques used for studying nematode community

1.1 Nematodes and their general ecology

Nematodes are small worm-like animals (usually 0.3–5.0 mm long as adults) and are considered to be the most abundant animals on Earth (Yeates, 1979; Bongers *et al.*, 1999). Although nematode species can be difficult to distinguish, over 25,000 have been described (Hodda, *et al.*, 2011; Zhang *et al.*, 2013), of which more than half are parasitic. The total number of nematode species has been estimated to be about 1 million (Lambshead, 1993).They often outnumber other animals in both individual and species counts. For example, they represent 90% of all animals on the ocean floor (Danovaro *et al.*, 2008).

Nematodes occupy almost any environment that provides an available source of organic carbon - from marine (salt water) to fresh water, to soils, and from the polar regions to the tropics, as well as the highest to the lowest of elevations (Yeates, 1987). Nematodes have been found in every part of the earth's lithosphere (Borgonie *et al.*, 2011), even at great depth (0.9–3.6 km) below the surface of the Earth in gold mines in South Africa (Borgonie *et al.*, 2011).

The numerical dominance of nematodes, and their presence at various trophic levels, leads to them playing a significant role in nearly all the world’s ecosystems (Platt, 1994). Nematodes take key positions as primary and intermediate consumers in soil food webs, and therefore they make good bioindicators (Bongers *et al.*, 1999). They occur in any environment that provides a source of organic carbon, in every soil type,

under all climatic conditions and in habitats that vary from pristine to extremely polluted. Nematodes occupy key positions in soil food webs. They feed on most soil organisms and are food for many others. They also influence vegetation succession. Nematodes respond rapidly to disturbance and enrichment: increasing microbial activity leads to changes in the proportion of bacterial feeders in a community. Therefore, studying nematode community and assessing the controls on diversity and structure of nematode community can be seen as being of great importance (Bongers *et al.*, 1999; Ritz *et al.*, 1999).

In soil, nematodes live in capillary water; their permeable cuticle provides direct contact with their microenvironment. They cannot rapidly migrate from stressful conditions and many species survive dehydration, freezing or oxygen stress (although others are more sensitive). The community structure of nematodes is thus indicative of conditions in the soil horizon that they inhabit.

Classically, nematodes have been identified morphologically. Because nematodes are transparent, their diagnostic internal features can be seen without dissection. There is a clear relationship between structure and function: the feeding behavior is easily deduced from the structure of the mouth cavity and pharynx.

1.2. Approaches used for studying nematode community

1.2.1 Importance of taxonomy

Understanding how an ecosystem works depends not only on holistic syntheses of all components but also on our knowledge of how its individual components function (Kotliar, 2000). Accuracy of identification is, therefore, fundamental to our understanding and communication of the ecological role of any organism. Hugot (2002) emphasized the need for correct identification and the role of taxonomy in science. The current need for a revamped effort to address the gap between estimated and documented nematode species diversity cannot be over-emphasized (Hugot, 2002). Traditionally, nematology has its strength in agricultural applications because of its economic implications. As a result, nematode species delimitation methods in the context of agricultural and health-related applications are more refined at the species and below species level than methods employed in broader nematode biodiversity studies. The biodiversity/ecological side of nematode taxonomy, which often deals with free-living forms, remains relatively lacking in research input.

1.2.2 Classification of nematodes by morphological observation

A classification scheme can be a useful and valuable way to group organisms in order to visualize the similarities and differences between them. If one knows the characteristics of one nematode species, one likely also knows a considerable amount about closely related nematodes in the same taxonomic grouping. Surveying various textbooks on parasitology and nematology, considerable agreement can be found as to the nematode species which are the most important parasites. Most of these sources are also in agreement as to the highest (class) and lowest (genus and species) levels of classification of phylum Nematoda. However, when it comes to intermediate levels there is very little consistency in classification. There is general agreement as to which nematodes should be grouped closer together, but not to the taxonomic level to which to assign the group or to the number of subgroups into which they should be divided. Some of this results from the previously mentioned division between helminthology and nematology and some from the fact that this is still an active area of research, generating different perspectives and opinions.

The biological definition of species based on inter-fertility has very little application in the classification of nematodes. Classification of the thousands of species that have been described is based largely on morphology, life history, and habitat. The life cycles and breeding

habits of many nematodes are unknown.

1.2.3 Classification of nematodes by molecular methods

Recently, the phylogenetic species concept – based on history of descent and differences in DNA sequences - has gained more support in nematode classification (Adams, 1998, 2002). Ways to extend its theoretical appeal into practicality have been evaluated (Nadler, 2002). Molecular methods have a number of advantages:

1) They can be applied to whole mixed communities of nematodes using high throughput systems. Thousands of sequences of genetic information in the form of DNA sequences of one or a few genes can be acquired for the whole nematode community, in a few days. This makes the study of nematodes much less labor intensive than was the case before, when morphology had to be used. Minor technical limitations (e.g., in extraction, finding broadly applicable primers etc.) remain because of the differing techniques needed to isolate nematodes from substrate in different environments.

2) Homology of genes is simpler to predict and test than homology of morphological characters. In addition, both types of data can be used to infer phylogenetic relatedness and thus create a systematic framework in an evolutionary context. However, because of their sheer amount of sequence data and simple methods for predicting homology based on DNA sequences (Schwarz, 2005), molecular characters

rapidly outnumber morphological ones. Inferring phylogeny from DNA sequences, similar to morphology, has its own recognized methodological limitations that may affect the conclusions. Alignment of sequences using computer algorithms may introduce biases, especially when they are subsequently modified by eye – however, deciding positional homology of DNA sequences is a more consistently applicable process than similar decisions for morphological characters.

3) DNA sequences are already digital and can be communicated readily across laboratories without interpretation.

4) DNA sequences are inherently genetic and therefore overcome many problems of apparent similarity from which morphology suffers, especially with microscopic organisms. Analysis of DNA sequences has unveiled cryptic species among individuals considered conspecific based on morphology alone (Eyualem and Blaxter, 2003; Hoberg *et al.*, 1999; Chilton *et al.*, 1995; Nadler, 2002; Fonseca *et al.*, 2008; Derycke *et al.*, 2008; Bhadury *et al.*, 2008).

1.2.4 Comparing approaches for studying nematode communities

Ecological research generally requires a community level analysis, as opposed to using single taxa or functional groups, as studies have shown that discrimination between treatments is greatly enhanced by analyzing the community (Freckman *et al.*, 1993) and other microbial

or biochemical indicators (Ettema *et al.*, 1999). Although one of the perceived advantages of using nematodes as an ecological indicator is the existence of readily identifiable functional guilds (Yeates *et al.*, 1999), identification to the species level is problematical in many cases. Some species cannot be distinguished morphologically from routine soil samples, certain rhabditid nematodes, for example, can only be differentiated from males which can form less than 0.1% of the population (Vanderknapp *et al.*, 1993). The specialised taxonomic input required to compile species lists of, for example, the 154 nematode species recorded from an English grassland (Hodda *et al.*, 1994) or 113 species from a German stream (Beier *et al.*, 2003) is considerable and means that most studies are conducted at a less detailed taxonomic level (Yeates *et al.*, 1999). The severe shortage of taxonomically competent persons, especially for microbial-feeding nematodes, has been recognized (Bernard, 1992; Coomans, 2002). In summary, morphological taxonomic identification to species level is often not possible due to the absolute number of nematodes to be identified, a lack of specialist knowledge and the fact that most species can only be identified from adult characters (Floyd *et al.*, 2002). The majority of community analyses, therefore, have been carried out at the genus, family or functional guild level (Bongers *et al.*, 1999). It has also been stated, however, that a species level analysis is more meaningful and should be preferred (Bernard, 1992) and is necessary to permit further advances in understanding the role of nematodes in soil processes

(Yeates, 2003).

The taxonomy bottleneck to nematode community analysis could potentially be overcome using molecular biological techniques. Ritz and Trudgill (1999) recognized the need for an approach that did not require extensive taxonomic skills, although they advocated a functional guild rather than molecular approach. Yeates and Bongers (1999) stated that molecular techniques would need to reflect the relative abundances of the particular species or functional guilds. Vanderknapp *et al.* (1993) used an arbitrarily primed PCR technique to differentiate closely related bacterial-feeding nematode species (from agar culture) that could not be morphologically distinguished, and suggested that the technique could be used in an ecological context. It would, however, require PCR-amplification of individual nematodes with at least three different primer sets and could not identify the nematodes without considerable calibration. Eyualem and Blaxter (2003) were able to characterize five morphologically identical populations of Panagrolaimus (again from agar culture) into two species by sequencing 18S rDNA. Floyd *et al.* (2002) developed a 'molecular operational taxonomic unit' approach in which PCR-amplification products from individual nematodes (both from agar culture and field samples) were sequenced and suggested modifications to make the method applicable to community analysis. Foucher and Wilson (2002) used denaturing gradient gel electrophoresis (DGGE), to distinguish nematode species present in a mixed laboratory culture, and

suggested that the method had advantages, over a morphological analysis, of speed and obviated the requirement for taxonomic skills. Waite *et al.* (2003) also used DGGE to analyze nematode communities from DNA directly extracted from a single gram of soil. This latter approach gave a community 'fingerprint' which differed between sites, but from which it was not possible to infer much about the genetic diversity or relative abundance of any particular taxa. Foucher *et al.* (2004) subsequently used DGGE to assess nematode biodiversity, but similarly without making any analysis of the taxa present or their abundance from the molecular data.

These approaches to nematode community analysis using molecular techniques have addressed some but not all of the difficulties associated with morphological analysis as outlined above. The requirements of a molecular approach are: (1) efficient extraction of DNA that is representative of the community (either direct extraction from soil or from extracted nematodes); (2) that the output should provide a quantitative (or semi-quantitative) measure of identified taxa to allow for a functional analysis of the community and (3) the ability to process large numbers of samples. Blaxter (2004) recognised the advantages of high-throughput, sequence-based molecular taxonomy, both for single specimens and for communities.

CHAPTER 2
The diversity of soil nematodes maximized in mid-elevations on Mt. Norikura

2.1. Introduction

2.1.1. Elevational change of climate and abiotic factors

Several factors change predictably with increasing elevation; the most obvious of which is the generally linear decrease in temperature. Temperature decreases by an average of approximately 0.68 C for each 100 m increase in elevation (Barry, 2008). This is termed the moist adiabatic lapse rate and varies depending on the latitude, size, shape and prevailing weather patterns on the mountain from 0.48 C to 0.78 C for each 100 m increase in elevation (Barry, 2008). Tropical mountains, due to higher temperatures at low latitudes, have warmer temperatures at the base and therefore need to be much taller to reach the extreme cold temperatures seen on temperate mountains. Other abiotic factors that vary predictably with elevation are air pressure, which decreases with increasing elevation, and solar radiation, which increases with increasing elevation (Barry, 2008).

Other climatic and abiotic factors vary along montane gradients but have a more complex relationship to elevation. The best example of such a factor, and probably most important, is precipitation (Barry, 2008). Precipitation can be in the form of rain, snow and condensation from clouds (e.g., horizontal precipitation in cloud forests). The elevational precipitation trends tend to correspond to the prevailing weather patterns, the slope and the proximity to the ocean or a large water body (Barry, 2008). The most common elevational pattern is

increasing precipitation with increasing elevation. This pattern predominates on mountains at temperate latitudes, and in arid regions regardless of latitude (Barry, 2008). Tropical mountains show a more variable pattern and display decreasing trends, unimodal or bimodal trends with highest precipitation at middle elevations and increasing trends. Some mountains (e.g., in Vietnam or French Guiana) show little variation in precipitation across the elevational gradient. The interaction among temperature, precipitation, cloud cover and solar radiation determine the overall primary productivity of each elevation and this, like precipitation alone, displays elevational trends which vary considerably among mountains. Other abiotic factors which vary with elevation and can be important determinants of species richness include area, cloud cover and soil quality, among others. For example, montane cloud forest is linked to small mammal diversity patterns and is a habitat predominantly created through the tendency of cloud cover to persist at mid- to high-elevations. Montane cloud forests vary in elevation among mountains depending on the distance to the ocean, mountain height and local climatic conditions such as prevailing winds and local temperature or precipitation regimes.

2.1.2. History of research on elevational species richness

The reduced number of species of plants and animals on mountaintops in comparison with the plethora of species in the

lowlands was no doubt known to the earliest human societies (Lomolino, 2001). By the nineteenth century, early naturalists including Linnaeus, Willdenow, von Humboldt, Darwin and Wallace noted that species richness decreased from low to high latitudes. And on their explorations of tropical mountains – von Humboldt and Darwin predominately in the South American Andes, and Wallace in Southeast Asian islands – noted the same trend for elevation; the number of species appeared to decrease from low to high elevations (Lomolino, 2001). It was not until the twentieth century that quantitative assessments were compiled to evaluate elevational trends in species richness. Joseph Grinnell, a vertebrate biologist at the newly founded Museum of Vertebrate Zoology at the University of California Berkeley, set out to detail the elevational distributions of terrestrial vertebrates on various mountains in California. This work is best known for his conceptual delineation of the niche (Grinnell, 1917), but in his studies the earliest recorded elevational richness patterns can also be found for the Yosemite and Lassen elevational gradients among others (Grinnell *et al.*, 1924; Grinnell *et al.*, 1930).

Grinnell and Storer (1924) determined that each group of vertebrates on the Yosemite transect (bats, nonflying small mammals, breeding birds, amphibians and reptiles) exhibited a unimodal richness pattern with the highest species richness about a third of the way up the mountain. In contrast on Lassen, Grinnell and colleagues found that non-flying small mammals and birds had the highest number of species

at mid-elevations, whereas reptiles and bats had the highest richness at the lowest elevations (Grinnell *et al.*, 1930). Later in the century, Robert Whittaker and colleagues set about describing how insect and plant diversity changed along elevational gradients on various mountains in the United States (Whittaker, 1952, 1960). Whittaker, like Grinnell, described two elevational patterns of species richness – decreasing with elevation and mid elevational richness peaks that varied among mountains and organism of study (e.g., trees, bushes and herbs; flies, beetles and grasshoppers).

In the 1970s and 1980s, ecologists became enamored by the extraordinary diversity of the tropics, and attention shifted away from temperate elevational studies. The first tropical elevational gradient study to make a decisive mark on the research community was John Terborgh and colleagues' examination of bird communities in the Peruvian Andes (Terborgh and Weske, 1975; Terborgh, 1977, 1985). Like Darwin and Wallace, Terborgh detailed decreasing richness with increasing elevation in Peruvian birds and he noted the strong parallel between the latitudinal and elevational gradients in diversity. Based on these results, decreasing elevational diversity became the accepted and assumed pattern for all taxonomic groups for more than two decades (Brown and Lomolino, 1998), and the unimodal elevational patterns of Grinnell and Whittaker were largely forgotten. The uniformity of decreasing richness on elevational gradients was challenged by Rahbek (1995). He showed that the decreasing trend in richness found by

Terborgh was only from nonstandardised samples, and when samples were standardised (Terborgh, 1977) a mid-elevational peak occurred. Rahbek (1995) also presented a series of case studies emphasizing that unimodal trends were more common than decreasing patterns. Recently, there has been a concerted effort to systematically document species richness along elevational gradients around the world for many groups of plants and animals (Heaney, 2001; Kessler *et al.*, 2001; Sanders, 2002; Brehm *et al.*, 2003; Grytnes, 2003; Herzog *et al.*, 2005). Additionally, a series of systematically compiled metaanalyses were conducted for various taxonomic groups (McCain, 2005, 2007b, 2009, 2010).

2.1.3. Patterns in Species Richness with Elevation

Elevational patterns in species richness fall into four common patterns: decreasing, low plateau, low plateau with a mid-elevational peak and mid-elevation peak (McCain, 2009). These have been variously defined and named, but here we follow the quantifiable definitions of McCain (2009). Decreasing richness patterns are those in which species numbers decline generally monotonically with increasing elevation. Low plateau patterns have consecutively high richness across the lower portion of the gradient and thereafter decreasing species richness. Low plateau patterns with a mid-elevational peak have high richness across low elevations with a diversity maximum found more

than 300 m from the base. Mid-elevation peaks have a unimodal peak in diversity at intermediate elevations with 25% or more species than at the base and top of the mountain. Rarely, species richness increases with elevation (*e.g.*, for salamanders and lichens in Martin, 1958; Wake et al., 1992; Grytnes *et al.*, 2006). As displayed in the early studies of Grinnell and Whittaker, the patterns of elevational species richness reflect the ecology of the particular taxonomic group (McCain, 2009, 2010). Meta-analyses of terrestrial vertebrate groups found that the predominance of a particular elevational pattern of species richness was clearly linked to taxon. Nonflying small mammals almost ubiquitously display mid-elevational peaks in diversity (McCain, 2005), whereas bat elevational patterns were evenly split between decreasing and mid-elevational peaks (McCain, 2007b). Birds and reptiles displayed all four common patterns of elevational species richness – evenly for birds (McCain, 2009), and with a predominance of decreasing patterns for reptiles (McCain, 2010). Preliminary analyses for amphibians show that salamanders displayed mostly mid-elevational peaks in species richness, whereas frogs showed all four common patterns in similar frequency. Although no meta-analyses have been completed for plants and insects, the literature shows examples of all four patterns among various groups. Rahbek (2005) included many plant studies in his overview of scale and species richness, and found most displayed mid-elevational peaks along elevational gradients. There is almost no documentation of elevational patterns of microbe diversity, although

one study found a decreasing taxon diversity pattern for bacteria in the Rocky Mountains of Colorado between 2460 and 3380 m (Bryant *et al.*, 2008).

2.1.4. Nematode community in relation to elevational gradient

There has so far been very little concerted effort to determine how diversity patterns and community structure of soil nematodes vary with elevation, despite their abundance and important roles in ecosystem processes (Bongers *et al.*, 1999).To date, only one study has focused on diversity patterns and community structure of soil nematodes vary with elevation, finding no evidence of changes in nematode community diversity across a 1,618 m range of elevation in the USA (Treonis *et al.*, 2012). However, this study used low-resolution TRFLP molecular techniques, greatly limiting its applicability to a study of diversity and community structure. Given the lack of existing studies of soil nematode communities in relation to elevational gradients, it is important to add new work on mountain systems to understanding what patterns exist and what environmental factors may cause these patterns. As well as being of interest in the study of nematode ecology, this would be an important contribution to arriving at general patterns and principles in ecology.

2.1.5. Questions and hypothesis

Here, we set out to conduct systematic and representative study focusing on soil nematodes, using high resolution molecular techniques over a broad elevational range. Our predictions were:

1) Diversity of soil nematode communities will differ with elevation, showing a mid-elevation maximum. Reviewing studies of elevational diversity trends on a broad range of other taxa, Rahbek (1995, 2005) found that around half showed a mid-elevation maximum. Since this is the most common elevational pattern, it is most likely that nematode community will also follow the same pattern, showing a diversity maximum at mid-elevations. We were firstly interested in knowing whether this empirical pattern is still more widespread, occurring in yet another group of organisms. Moreover, we anticipated that the tendency for other groups of organisms to show mid elevation maxima might actually interact with nematode ecology to produce this pattern in nematodes. If the diversity of trophic sources is greater in mid elevations - due to greater taxonomic diversity in a group that is a food source - this can be expected to allow a greater number of nematode niches to coexist. No relevant studies of elevational trends in other groups of soil organisms exist for Norikura, with the exception of archaea which also show a mid-elevation diversity maximum on Norikura (Singh, 2012). However, on nearby Mt. Fuji, which has a very similar climate and reaches a similar height to Mt. Norikura, there are

mid elevation diversity maxima in both bacteria (Singh, 2012) and fungi (Miyamoto, 2014), as well as archaea (Singh, 2012). As these are groups that many soil nematodes feed on (Yeates, 1993), the greater diversity of these food sources could hypothetically contribute to a mid-elevation maximum in nematode diversity.

A further reason to anticipate a mid-elevation diversity maximum for nematodes is the predominance of a mid-elevation maximum in precipitation across high mountains in central Japan (Fujimura, 1971). For example, Mt. Fuji has an annual precipitation maximum at 2,000-2,500 masl (Fujimara, 1971). This coincides with the elevation of the predominant cloud layer, and although the elevational trend of precipitation on Norikura has not been measured, the cloud layer likewise tends to occur at around 2,000-2,500 masl (pers. obvns. by the authors). A mid elevation precipitation maximum for Norikura is potentially significant for soil nematodes because they are strongly dependent on moving within the soil water that surrounds and fills spaces between soil particles (Stirling, 2014). Greater precipitation, combined with cooler temperatures in mid elevations, may be expected to give moister – but still aerated - soils that are physiologically favorable to nematodes and their feeding activity. This itself may promote greater diversity of nematodes because there are fewer restrictions of survival and feeding, allowing greater overall population densities even in specialized niches, promoting niche specialization, and thus species diversity. The precipitation/water balance maximum in

mid elevations has been suggested as a cause of diversity maxima in other parts of the world, in other groups that are particularly sensitive to water balance (Wolf, 1993; Olson, 1994).

There has been much discussion of the possible reasons why mid-elevational diversity maxima occur so commonly (Colwell, 2000; McCain, 2004; Lomolino, 2001). Apart from the potential role of mid-elevation precipitation maxima, one widely favored explanation for the mid elevation diversity maximum is the 'community overlap' hypothesis, which assumes that there are often two relatively distinct environments on a mountain – an 'upper mountain environment subject to more extreme low temperatures or a different precipitation regime (and often above the treeline), and a 'lower mountain' environment that has a distinct ecology (and is often by contrast forested) (Lomolino, 2001). If each of these two environments has its own distinct set of species, at the transitional zone where they intersect both groups of species will occur together or nearby one another, giving a mid-elevation diversity maximum (Lomolino, 2001).

Another explanation for mid elevation diversity maxima is the 'mid-domain effect' (MDE) (Colwell, 2000), which simulates the interaction between a unimodal gradient in favorability with elevation, and the geometric constraints that are imposed by domain limits. MDE states that if all species ranges are scattered randomly between the limits of the top and bottom of a mountain, there will be a 'bulge' of maximum numbers of overlapping species in the mid elevations

(Colwell, 2016). Besides testing these predictions for overall trends on mountains, we were also interested in a more generalized way in understanding which particular environmental variables are contribute to whatever elevational diversity trend exists, since studies have shown that nematode communities are sensitive to the surrounding environment and several environmental variables. For example, salinity, temperature, pH, soil moisture and organic nutrients have been identified as important factors influencing diversity of nematode communities (Powers, 1998, Freckman, 1997).

2) Along elevational gradients, community structure of soil nematodes will differ in relation to elevation, producing a clear succession of communities. It is known that for many taxa, there are distinct and predictable community assemblages of organisms which occur at particular elevational levels on mountains (Patterson, 1998; McCoy, 1990; Singh, 2014). This is a result of the fact that many types of organism have clearly defined spatial ranges that are linked to climate and the ecosystem conditions that parallel elevation. The overlap of these ranges is what produces the characteristic communities of each elevational zone. Given that this elevational turnover pattern in species and communities is so widespread, we hypothesized that nematodes, too, would have finely adapted environmental niches in relation to particular elevations, and that this would produce a series of distinctive communities along the elevational gradient.

3) That nematode range extents will follow Rapoport's elevational

rule. We were also interested in testing for the existence of a pattern which has been reported from a range of studies: Rapoport's elevational rule. This is a derivative of Rapoport's rule [Stevens, 1989], which notes that higher latitude species tend to have larger geographical ranges. The elevational variant of this rule predicts that species found at higher elevations will tend to occur over a broader range of elevations than those from lower down the mountain (Stevens, 1992). The pattern has been documented along elevational gradients in a variety of different taxa, and it has been suggested that it occurs because climates at higher elevations are more variable, so species that occur there must be more tolerant of temperature variation - which also translates into larger elevational ranges (Stevens, 1992).

2.2. Materials and methods

2.2.1. Description of sampling sites

Mt. Norikura is an extinct volcano in central Japan. It reaches 3,026 masl and has a cool temperate monsoon climate at its base (MAT of 8.5 ˚C at 1,000 masl) with abundant year-round precipitation (2,206 mm at 1,000 masl) concentrated in summer (Miyajima *et. al.*2007; Takahashi *et. al.*2003). Its surface covering of soils is uniformly derived from andesitic ash deposited in a series of large eruptions around 15,000 years ago (Miyajima *et. al.*2007; Takahashi *et. al.*2003). Mt. Norikura has since then undergone natural ecological succession to

give a series of vegetation zones.

Three major vegetation zones of tree species are recognized between 800 and 3,000 masl. on Mount Norikura—a montane deciduous broad-leaved forest zone between 800 and 1,600 masl, a subalpine coniferous forest zone between 1,600 and 2,500 masl, and an alpine dwarf pine *Pinus pumila* scrub zone between 2,500 and 3,000 masl. The timberline was at approximately 2,500 masl on the east slope of Mount Norikura (Takahashi, 2003) studied in this work. Kira's warmth index (*WI*), is often used to express relationships between thermal conditions and vegetation (Kira, 1948). The *WI* expresses the approximate effective heat for plant growth and is calculated as $\sum(m_t - 5)$, where m_t is mean monthly temperature above 5 C. The *WI* was estimated as 48.5 C months at 1,600 masl (the upper distribution limit of the montane broad-leaved forest zone) and 21.9 C months at 2,500 masl (the timberline) on Mount Norikura by using decadal temperature data (1994–2003) recorded by Nagawa weather station, with a lapse rate of −0.55 C for each +100 m in altitude.

Although vegetation between 800 and 1,600 masl was partly subjected to anthropogenic effects, this study was performed at sites without the anthropogenic effects. Anthropogenic effects on vegetation were negligible from 1,600 masl to the summit. Dominant species were deciduous broad-leaved *Zelkovaserrata*, *Juglans mandshurica* var. *sachalinensis*, and *Lindera praecox* at 800 masl, deciduous broad-leaved *Quercus crispula*, *Castanea crenata*, and*Betula*

platyphylla var. *japonica* at 1,400 masl, evergreen conifer *Abies veitchii* and *Tsuga diversifolia* at 1,600–2,000 masl, and evergreen conifer *Abies mariesii*, and deciduous broad-leaved *Betula ermanii* and *Sorbus matsumurana* at 2,200–2,500 masl, Plant nomenclature is in accordance with Shimizu (1997) (Fig. 1-5).

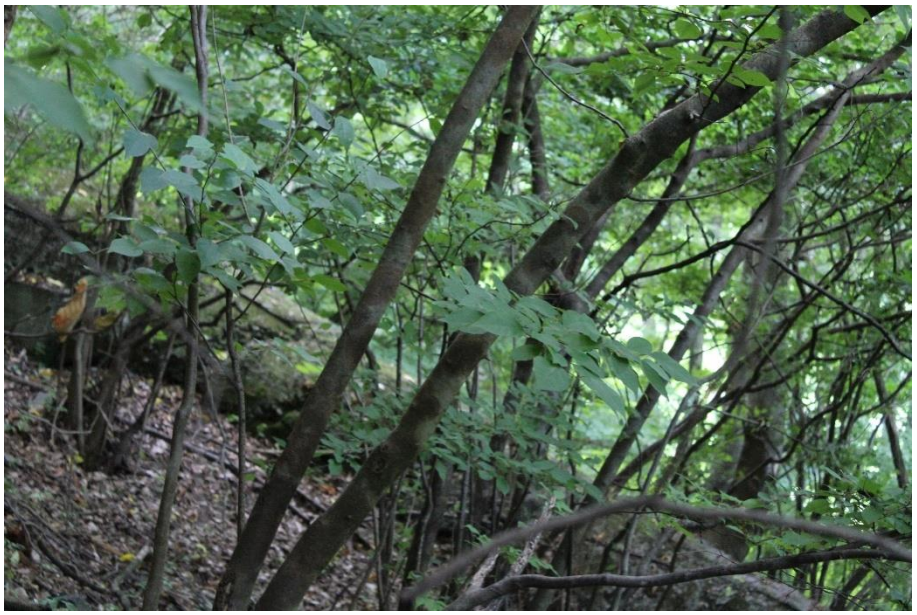

Fig. 1 Photos of sampling sites on Mountain Norikura at ~700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Fig. 2 Photos of sampling sites on Mountain Norikura at ~1,000 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Fig. 3 Photos of sampling sites on Mountain Norikura at ~1,700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Fig. 4 Photos of sampling sites on Mountain Norikura at ~2,300 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Fig. 5 Photos of sampling sites on Mountain Norikura at ~2,700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

2.2.2. Sampling and soil analysis

Sampling was carried out over ten days from late July to early August 2014. In total, 55 samples were collected from 11 elevations on the mountain, along elevational isoclines bands separated by ~200 m of elevation. At each elevational level, five separate samples that consisted of a 10 m by 10 m quadrat were sampled (Fig. 6; Table 1). At each elevational level, the quadrats were spaced 100 m apart in a line that followed the elevational contour (measured by GPS).Soil cores 10 cm in diameter and 10 cm deep were taken at the four corners and central point of each quadrat. Soil from the four corners and center of each quadrat was then combined into a single sampling bag. The sample bags were transported to the laboratory at Shinsu University within 5 hours of being gathered. At the laboratory, the contents of each bag were gently mixed, then gently passed through a 5 mm sieve.

Nematodes were collected by a modified Baermann funnel method, with nematodes travelling downwards through waterlogged soil and being collected in a tube below (Van Bezooijen, 2006). Immediately on arrival of the soil sample at the laboratory, 200 g of soil was wrapped loosely with 2 mm medical gauze and placed into a funnel, which was then filled with distilled water at 20 ˚C, with the apparatus kept in an air conditioned room at 20 ˚C during extraction. After 24 hours, 30 ml water in the bottom of the funnel, containing both, nematodes and soil particles was collected to centrifuge and the sediment was used to

extract nematode DNA.

The Baermann funnel is used for extraction of active nematodes from plant material and soil. The sample size depends on the funnel diameter and the type of material. If extraction is from soil, the final suspension is dirty. The method makes use of nematode mobility. If material is placed in water, nematodes crawl out of the material and sink. The method is simple and cheap and when extracting nematodes from plant material, the final suspension is virtually clean. The extraction efficiency is rather high for small samples, but for larger samples efficiency decreases exponentially. Many nematodes die due to accumulation of metabolic coreucts and microorganisms, and lack of oxygen at the bottom of the funnel. To inhibit bacterial growth add a few ml of methyl-p-hydroxybenzoate (0.15%) to the water and to avoid lack of oxygen use a 0.15% solution of hydrogen peroxide instead of water (Baermann, 1917).

Soil analysis of each sample was carried out at Shinsu University, using standard SSSA protocols (Soil Science Society of America). The parameters analyzed were total carbon (TC), total nitrogen (TN), available phosphate (P_2O_5), nitrogen in ammonium (NH_4-N), nitrogen in nitrate (NO_3-N), potassium (K), pH and soil texture. Total percentage silt and clay content are used to indicate soil texture in this paper. Mean annual temperature (MAT) was calculated by using a mean lapse rate of 0.6 ˚C /100 masl, so it showed a complete correlation with elevation.

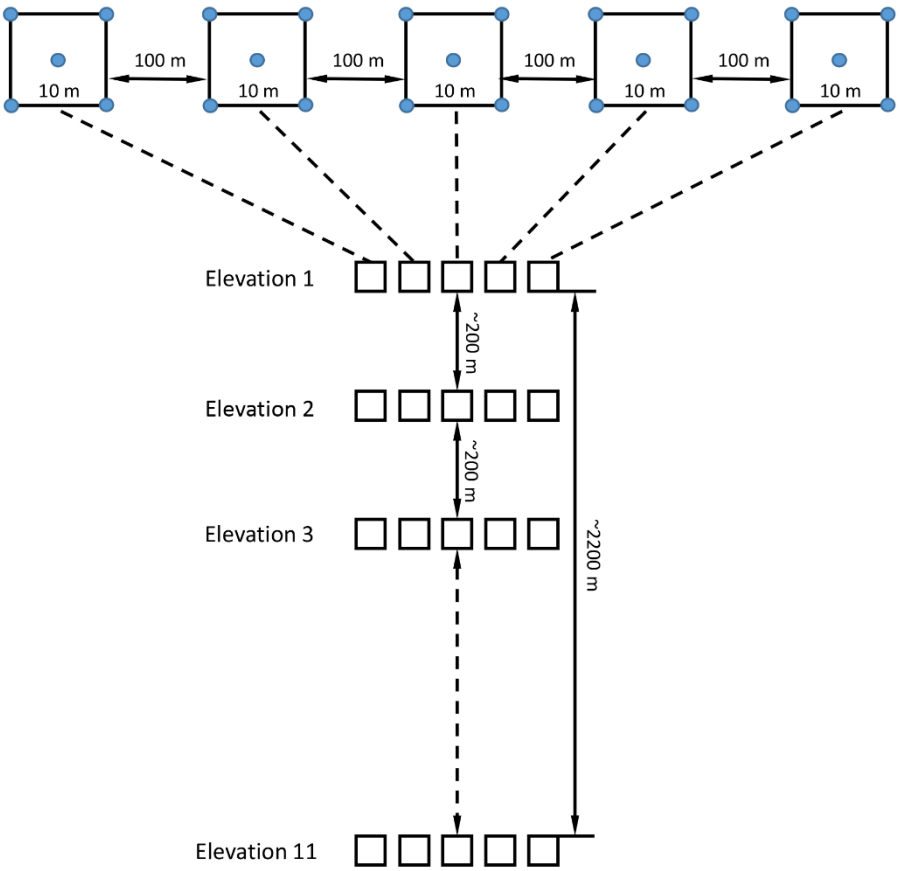

Fig. 6 Sampling scheme. Samples were collected from 11 elevations of the mountain, separated by about 200 m elevation. At each elevational level, five quadrats (10 x 10 m in size) were collected 100 m apart along a linear transect. Soil collected from the four corners and center of the each quadrat was pooled to make one samples for DNA extraction and soil property analysis.

Table 1 The sites sampled on Mountain Norikura. GPS coordinate was used to label the sampling site of each sample.

Sample name	N	E	Elevation (masl)
N1	36.109861	137.55225	2934
N2	36.109583	137.552778	2934
N3	36.109806	137.552194	2946
N4	36.109083	137.553667	2941
N5	36.109806	137.550083	2942
N6	36.123333	137.555778	2739
N7	36.123111	137.558056	2740
N8	36.122889	137.558083	2738
N11	36.114	137.567889	2522
N12	36.114278	137.567944	2518
N13	36.114611	137.567833	2520
N14	36.114722	137.567583	2521
N15	36.114444	137.567333	2522
N16	36.119333	137.5715	2360
N17	36.119222	137.572028	2351
N18	36.119222	137.572333	2337
N20	36.119722	137.5705	2378
N21	36.121972	137.583583	2027
N22	36.121972	137.585167	2090
N23	36.12225	137.584556	2051
N24	36.121972	137.584194	2057
N25	36.122028	137.583694	2070
N27	36.106833	137.608806	1691
N28	36.106667	137.608333	1696
N29	36.106361	137.605722	1700
N30	36.106139	137.607556	1707
N31	36.118306	137.624333	1492
N32	36.118611	137.622389	1487
N33	36.116667	137.623667	1492

N34	36.118778	137.623278	1500
N35	36.118333	137.623722	1489
N36	36.127056	137.650722	1319
N37	36.126944	137.650806	1321
N38	36.126833	137.650806	1321
N39	36.126917	137.650778	1320
N40	36.125556	137.650667	1318
N41	36.133306	137.673028	1100
N42	36.133583	137.672778	1105
N43	36.133306	137.672806	1095
N44	36.133583	137.672694	1103
N45	36.133472	137.672917	1105
N46	36.144167	137.744556	917
N47	36.008389	137.744417	960
N48	36.010889	137.743972	991
N49	36.010889	137.744083	990
N50	36.008583	137.744056	984
N51	36.180806	137.79425	744
N52	36.180667	137.794194	745
N53	36.180444	137.794361	744
N54	36.180667	137.794194	741
N55	36.180694	137.794472	750

2.2.3. DNA extraction, amplification, sequencing and quality control of 18S rRNA gene

DNA was extracted using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer's instructions, and random empty vials were chosen and run to serve as controls. The isolated DNA was stored at -80 ˚C until the PCR stage.

Miseq sequencing was used to assess the nematode community through massively parallel sequencing of the 18S rRNA gene. Compared to the previous studies conducted using 454 sequencing platform (Porazinska *et. al.*2009), this generated a larger data volume, which could minimize sampling errors. A 350-bp region of the 18S small-subunit rRNA gene was amplified with the primer pairs NF1 and 18Sr2b (Porazinska *et. al.*2009) with adapter sequences for the Illumina MiSeq. PCR was performed in a 50-µl reaction mixture composed of 1 or 2 µl of DNA extract, 0.4 µM of each primer, 0.2mM of each dNTP mix, 1X Taq Reaction Buffer and 1.25 U Solg™ Taq DNA Polymerase (SolGent co., Ltd., Korea). PCR conditions were 10 min at 95°C, followed by 30 cycles of 60 s at 95°C, 45 s at 50°C, and 180 s at 72°C. Final elongation was at 72°C for 10 min using the C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Index PCR was performed for the purified PCR products using the Nextera XT Index kit (Illumina, Inc., San Diego, CA, USA). Each of the 50-µl reaction mixtures was composed of 5 µl of each index primer,

2× PCR Solution Premix Taq™ DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), and 5 μl of the purified DNA. PCR conditions were 3 min at 95°C, followed by 10 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Final elongation was at 72°C for 5 min.

After purification by the AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA), each amplicon was normalized to 4 nM with 10 mM Tris–HCl (pH = 8.5) and pooled with an internal control PhiX (30%). Heat-denatured pooled amplicons were loaded to a v3 600 cycle-kit reagent cartridge (Illumina, Inc.), and 2 × 300 bp paired-end sequencing was performed by the Illumina MiSeq at the Graduate School of Public Health, Seoul National University.

The sequences were demultiplexed and trimmed with a read quality score above 20 by the MiSeq Reporter v2.5 (Illumina, Inc.). The sequence data obtained was processed following the Miseq SOP in Mothur (Schloss *et al.* 2009). Sequences with any ambiguous bases, sequences with more than 8 homopolymers and sequences with lengths less than 200 bp were removed using the screen.seqs command in Mothur. Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within Mothur in de novo mode. Rare sequences (less than 10 reads) were removed to avoid the risk of including spurious reads generated by sequencing errors (Brown *et al.* 2015).

The analysis of nematode richness/diversity and community structure of soil nematodes was conducted separately three times,

respectively setting the cutoff points of OTUs at 99%, 98% and 97% sequence similarity. These different sequence similarity levels were used to study the possible impacts of methodological biases in OTU definition, as the average phylogenetic distance between different nematode species is unclear (Brown *et al.* 2015). At different sequence similarity level, different subsampling depths were applied according to the lowest read number generated by sequencing of samples. Samples with too low read numbers were removed from further analysis.

Taxonomic classification of each OTU was obtained by classifying alignments against SILVA Release 115 databases (Quast *et al.* 2013) using the classify command in Mothur at 80% cutoff with 1000 iterations. The Miseq sequence data used in this study are deposited in the MG-RAST server (Meyer *et al.*, 2008).

2.2.4. Statistical analysis

Diversity indices such as Shannon index and Faith's PD and OTU richness were calculated using the Mothur platform (Schloss *et al.* 2009). To assess the best fitting model of correlations between elevation and richness/diversity and environmental variables, linear and polynomial (quadratic) models were tried out using SigmaPlot v 10.0 (Systat Software, San Jose, CA). Model selection was carried out based on adjusted R^2 and RMSE (root mean square error). We used ANOVA for normal data and Kruskall-Wallis tests for non-normal data to test

whether physicochemical characteristics differed among different elevations.

To evaluate in more detail the effect of these environmental variables on OTU richness, Shannon Index and Faith's PD, we performed multiple regression analyses. Non-significant predictor variables were removed sequentially until only significant variables were left in the model. Before applying multiple regression to the dataset, we looked for redundant physicochemical characteristics using the Varclus procedure in the Hmisc package (SAS Institute, 1990) in the R platform. In the study of nematode community on Mt. Norikura, high correlation was found between TN and TC (Spearman's $\rho^2 \geq 0.91$), between TN and P_2O_5 (Spearman's $\rho^2 \geq 0.73$) and between TN and soil texture (Spearman's $\rho^2 \geq 0.61$; Fig. 7). Therefore, we removed TC, P_2O_5 and soil texture from the analysis, and used the remaining seven physicochemical characteristics, {i.e. TN, NH_4-N, NO_3-N, K, pH, MAT and C/N ratio} for multiple regression analyses.

We performed a Non-metric Multidimensional Scaling (NMDS) using Primer v6 to explore patterns in community structure using a Bray Curtis similarity matrix. Abundance data of OTUs were square root transformed to calculate the distance between soil nematode communities. Community structure was compared between elevations using ANOSIM with 999 random permutations (Clarke & Gorley 2006). Then we visualized the comparison between the elevational abundance of different phylotypes at OTU level by drawing a heatmap of 30

abundant OTUs. Heatmap was drawn using pheatmap package in R.

To individually assess the influence of each environmental variables, canonical correspondence analysis was used in CANOCO ver. 5. Forward selection was used to select significant explanatory variables with 999 permutations and only significant ($p < 0.05$) variables were included in the models.

Nestedness analysis was performed by BINMATNEST with default input parameters (Rodríguez-Gironés *et. al.* 2006) to test whether the community of one set samples tending to be a subset of the community present in another set of samples. Nested patterns are those in which the species composition of small assemblages is a nested subset of larger assemblages. The significance value of nestedness was tested using default input parameters and null model 3 which calculates the p-value for row and column totals following Geel *et. al.* (2015). The samples were re-ordered following the packed matrix order which indicates a high-to-low categorized nestedness.

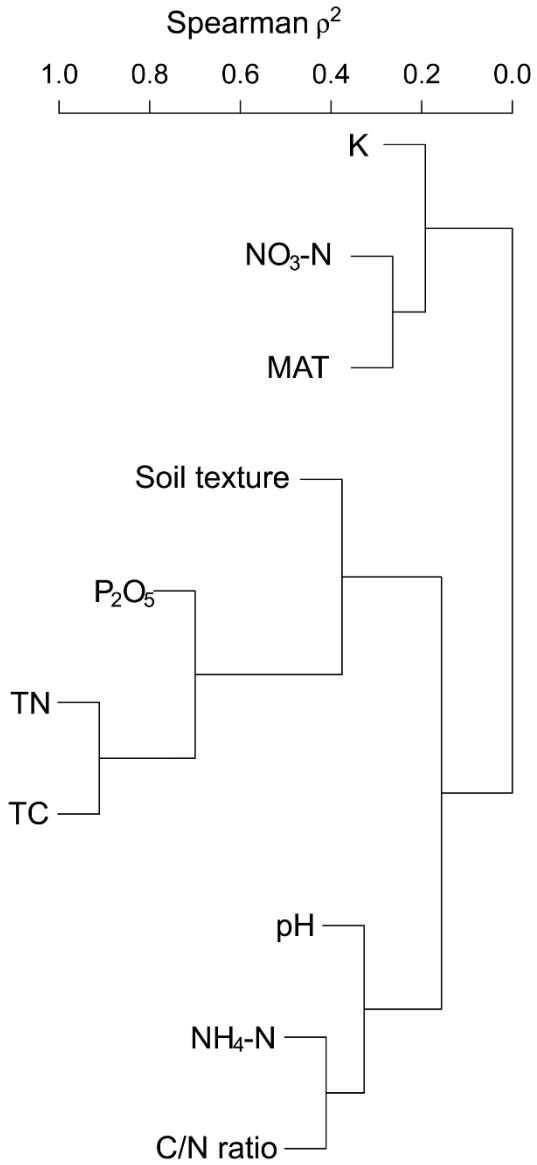

Fig. 7 Cluster analysis of all measured environmental variables.

2.2.5. Analyzing feeding groups of nematodes

We investigated the relative abundance of the dominant nematode families across all sites. Nematodes were identified to family and binned into their respective functional feeding guilds using previous published comprehensive lists (Yeates *et al.* 1993). Four feeding groups were created: (1) bacteria feeding, (2) fungi feeding, (3) plant feeding, and (4) omnivore/predator.

2.2.6. Defining elevational ranges of OTUs

We calculated the elevational ranges of OTUs following Colwell (Colwell *et al.* 2016). This range-adjustment procedures and assumptions have been widely used in previous studies (Colwell *et al.*2016). If the highest elevation at which an OTU was recorded was not at the highest sampling location, the upper boundary for that OTU range was estimated to occur halfway between the highest elevation of recorded occurrence and the next higher sampling elevation. If the highest elevation at which an OTU was recorded at was the highest sampling elevation, the upper boundary of that species range was estimated to occur halfway between that sampling elevation and the upper limit of the domain. The lower boundary for each range was treated analogously, being extended halfway to the next lower sampling elevation or halfway to the lower domain limit (sea level), if an OTU was recorded at the lowest sampling elevation, but that sampling

elevation was not the domain limit. The ranges of each OTU found at only one sampling elevation were treated similarly; otherwise, these point ranges would have had a zero range, and would have been lost from the model. We assumed that the occurrence of each species was continuous between its estimated upper and lower recorded range boundaries.

2.3. Results

2.3.1. Physicochemical characteristics

Nine physicochemical characteristics for the 55 samples were measured directly, or for elevation calculated on the basis of the moist air lapse rate, with the parameters being statistically different between elevations ($p < 0.01$) (Table 2). Mean annual temperature (MAT), which was conducted by using a mean lapse rate of 0.6 ˚C /100 masl, showed a complete correlation with elevation. The other physicochemical characteristics including total carbon (TC), total nitrogen (TN), available phosphate (P_2O_5), nitrogen in ammonium (NH_4-N), nitrogen in nitrate (NO_3-N), potassium (K), pH and soil texture all showed linear unimodal patterns against elevation (Fig. 8).

Table 2 Measured soil properties of samples collected along the ~2,200 meters elevational range on Mt. Norikura. Soil texture were shown as the percentage of silt and clay content. Mean annual temperature (MAT) was calculated by using a mean lapse rate of 0.6 ˚C /100 masl.

Sample name	Elevation (m)	Elevational isocline	Total N (%)	Total C (%)	pH	P2O5 (mg/L)	NH4-N (mg/L)	NO3-N (mg/L)	K (mg/L)	Soil texture	MAT
N1	2934	2940	0.07	1.07	6.05	0.45	5.90	7.68	0.00	18.24	-3.10
N2	2934	2940	0.04	0.44	5.63	0.00	6.83	6.78	0.00	16.51	-3.10
N3	2946	2940	0.01	0.15	5.89	0.00	6.52	4.97	0.52	17.15	-3.18
N4	2941	2940	0.02	0.16	5.81	0.00	3.26	2.03	0.00	16.83	-3.15
N5	2942	2940	0.03	0.20	5.55	0.00	5.35	4.75	0.00	24.12	-3.15
N6	2739	2740	0.07	1.41	5.52	0.45	7.92	7.46	0.00	22.03	-1.93
N7	2740	2740	0.05	0.58	5.58	0.45	3.72	3.39	0.00	27.21	-1.94
N8	2738	2740	0.06	0.72	5.50	0.00	5.12	3.39	0.00	25.24	-1.93
N9	2736	2740	0.09	1.61	5.53	0.00	4.42	6.10	0.00	22.28	-1.92
N10	2731	2740	0.13	2.27	5.82	0.22	7.45	12.88	0.99	24.91	-1.89
N11	2522	2520	1.71	27.15	4.91	8.07	52.77	36.61	0.00	61.96	-0.63
N12	2518	2520	0.31	5.28	5.56	0.00	14.90	21.02	0.00	33.22	-0.61
N13	2520	2520	0.85	14.57	5.60	0.45	18.16	99.44	0.00	43.87	-0.62
N14	2521	2520	0.93	16.66	5.23	0.67	33.52	5.65	0.00	52.32	-0.63

N15	2522	2520	0.16	3.33	5.58	0.00	12.11	11.53	0.90	34.79	-0.63
N16	2360	2360	0.91	16.99	5.11	1.87	19.79	27.12	1.50	57.59	0.34
N17	2351	2360	1.42	23.94	4.43	7.10	38.41	20.34	1.35	52.19	0.39
N18	2337	2360	1.02	19.66	4.54	6.72	4.66	4.52	1.30	61.09	0.48
N19	2350	2360	1.80	36.90	4.44	8.74	14.67	18.31	3.06	54.76	0.40
N20	2378	2360	2.11	33.38	4.62	10.76	42.60	34.58	0.00	52.00	0.23
N21	2027	2050	1.60	43.27	4.66	6.72	10.86	2.26	0.00	46.02	2.34
N22	2090	2050	1.70	42.78	4.20	52.07	47.49	0.00	0.00	38.33	1.96
N23	2051	2050	1.67	43.85	4.07	29.21	46.17	5.65	1.60	49.33	2.19
N24	2057	2050	1.51	44.02	4.84	20.54	42.68	9.94	3.08	42.48	2.16
N25	2070	2050	1.40	41.76	4.57	34.51	61.92	17.40	0.00	54.62	2.08
N26	1677	1700	1.38	31.25	4.70	10.08	13.27	10.17	2.43	62.62	4.44
N27	1691	1700	1.67	30.57	4.86	2.69	34.30	71.19	2.25	65.07	4.35
N28	1696	1700	1.61	32.12	4.40	17.48	44.00	16.27	2.61	51.81	4.32
N29	1700	1700	1.02	21.43	5.11	2.69	24.44	16.27	0.00	58.74	4.30
N30	1707	1700	1.53	30.97	4.84	12.77	30.03	10.17	0.00	44.23	4.26
N31	1492	1490	0.87	14.36	5.00	0.22	6.52	10.17	0.00	49.35	5.55
N32	1487	1490	0.85	15.51	5.22	0.00	16.06	4.07	0.00	45.52	5.58
N33	1492	1490	0.96	16.51	4.96	0.37	24.06	6.78	1.25	49.41	5.55
N34	1500	1490	0.80	14.11	5.15	0.22	21.34	4.07	0.00	33.45	5.50
N35	1489	1490	0.91	16.39	4.76	0.22	20.56	4.75	0.78	67.15	5.57
N36	1319	1320	0.69	12.88	5.35	0.00	11.87	3.39	1.05	25.71	6.59

N37	1321	1320	0.60	12.90	5.42	0.75	14.90	6.10	0.93	44.93	6.57
N38	1321	1320	0.58	11.07	5.59	0.37	15.52	4.52	1.30	32.63	6.57
N39	1320	1320	0.55	10.09	5.80	0.22	9.54	2.71	0.00	23.27	6.58
N40	1318	1320	0.82	14.95	5.41	0.22	14.20	17.63	0.84	36.45	6.59
N41	1100	1100	0.78	11.15	5.79	1.87	14.36	77.97	1.30	21.96	7.90
N42	1105	1100	1.44	21.28	6.07	6.72	18.62	99.67	1.60	27.62	7.87
N43	1095	1100	2.04	34.05	6.21	31.75	31.43	67.12	2.52	70.86	7.93
N44	1103	1100	0.62	8.74	6.41	0.45	6.29	49.72	0.99	31.12	7.88
N45	1105	1100	0.99	14.94	6.40	2.24	8.92	57.63	1.60	22.98	7.87
N46	917	950	0.84	10.98	6.31	0.45	4.66	57.63	1.02	48.09	9.00
N47	960	950	1.46	22.11	6.30	8.22	13.19	130.18	1.45	65.01	8.74
N48	991	950	0.80	10.10	6.00	0.45	3.72	53.11	0.81	37.39	8.55
N49	990	950	0.75	8.72	5.37	0.22	5.59	56.27	0.99	31.30	8.56
N50	984	950	1.17	15.32	5.88	0.90	7.45	76.84	1.23	55.89	8.60
N51	744	740	0.40	3.89	6.01	0.00	3.26	36.61	0.87	25.81	10.04
N52	745	740	0.90	10.85	6.09	0.45	4.42	77.97	0.96	34.10	10.03
N53	744	740	0.96	12.56	6.40	0.90	5.35	57.63	1.08	34.23	10.04
N54	741	740	0.68	6.89	5.26	0.22	4.19	35.93	0.99	22.94	10.05
N55	750	740	0.74	7.45	5.30	0.45	2.56	29.83	0.96	35.83	10.00

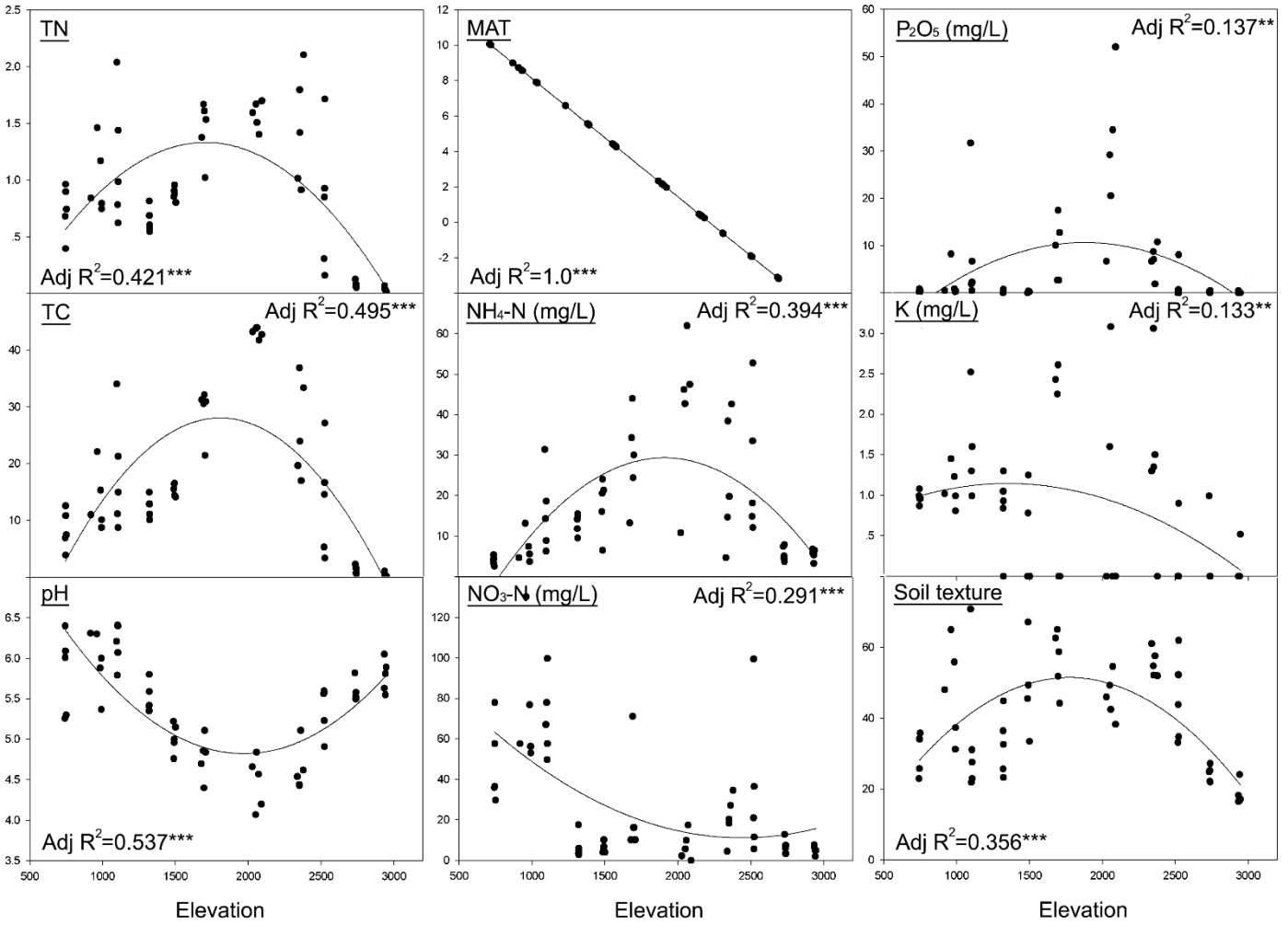
TN
Adj R2=0.421***
MAT
Adj R2=1.0***
P2O5 (mg/L)
Adj R2=0.137**
TC
Adj R2=0.495***
NH4-N (mg/L)
Adj R2=0.394***
K (mg/L)
Adj R2=0.133**
pH
Adj R2=0.537***
NO3-N (mg/L)
Adj R2=0.291***
Soil texture
Adj R2=0.356***
Elevation
Elevation
Elevation

Fig. 8 Physicochemical characteristics shown in relation to elevation. Model selection was carried out based on adjusted R^2 and root mean square error and only the significant ones are reported. The values of TC and TN are shown in percentages. Total of percentage silt and clay content are used to indicate soil texture.

2.3.2. Soil nematode community

In total, 55 samples were collected from 11 elevations on the mountain, along elevational isoclines bands separated by ~200 m of elevation, four of which were excluded from further nematode community analysis owing to failure of amplification of nematode 18S rRNA genes (Table 3). Only results conducted at a 99% sequence similarity cutoff level are shown, because analysis of OTUs at different sequence similarity levels showed similar patterns.

Along a ~2,200 meters elevational range, a total of 70,227 high quality 18S rRNA gene sequences were assigned to 1,121 operational taxonomic units (OTUs) at ≥99% similarity level from 51 samples after the removal of low quality, chimeric, and rare sequences. On average, 98.6 OTUs (±4.4 SE) were found in each sample. The greatest OTU richness was found at 1,105 meters above sea level (masl) with a mean of 149 OTUs per sample, while the lowest OTU richness was found at 2,941 masl with 22 OTUs per sample (Table 3 & 4).

The nematode community on Mt. Norikura were dominated by Prismatolaimidae which accounted for 15.3% of the total reads, followed by Mononchidae (10.7%), Qudsianematidae (10.6%), Rhabditidae (5.1%), Criconematidae (5.0%), Plectidae (4.9%), Chromadoridae (4.5%), Cephalobidae (3.9%) and Tripylidae (3.8%). The remaining 18.1% of the total reads were shared by 24 nematode

families together, and 17.9% of nematode sequences remained unclassified at the family level (Fig. 10).

Classifiable reads were binned into four feeding guilds (bacteria feeding guild [BF], fungi feeding guild [FF], plant feeding guild [PF] and omnivore/predator guild [OP]) according to their family taxonomy and all feeding guilds of nematode were found on Mt. Norikura (Fig. 11). BF and OP together accounted for the major portion of 72.3%, while FF were found at low abundances across all elevations of 2.4% in total. The percentage of PF was low on high elevations compared to the lower elevations below 2,500 masl (Fig. 11).

OTU richness, Shannon index and Faith's PD were used to assess the ecological pattern in nematode diversity. On Mt. Norikura, all of the diversity indices were significantly correlated with elevation ($p<0.001$), and the richness/diversity showed a mid-elevation maximum, with maximum diversity for all three indices falling between 1,000 and 2,000 masl (Fig. 9). Quadratic models gave the best fit for all three diversity indices. The greatest OTU richness was found at 1,105 meters above sea level (masl) with 149 OTUs, while the lowest OTU richness was found at 2,941 masl with 22 OTUs.

An NMDS performed on the Bray-Curtis similarity matrix of nematode community structure showed significant variability in relation to the elevational gradient (ANOSIM: Global $R = 0.761$, $p < 0.001$) (Fig. 12). The samples also formed clear clusters according to the elevation that they came from, visually indicating that samples

belonging to different elevational zones harbored distinct communities.

Nestedness analysis indicated that the communities of soil nematodes along the elevational gradient follow a nested structure ($p<<0.001$). A packed matrix order categorizing nestedness of each sample from low to high was generated, with samples from elevations above 2,500 masl intensively clustered on the top of the packed matrix order (Table 5). This indicates that the communities of higher elevations (above 2,500 m) tend to be a subset of the communities of lower elevations (below 2,500 m).

Table 3 Nematode reads generated by Miseq sequencing of each sample and sampling depth at 99% sequence similarity level.

Sample name	Nematode reads	Elevational isocline
N1	3955	2940
N2	8562	2940
N3	11140	2940
N4	11236	2940
N5	10632	2940
N6	5153	2740
N7	6381	2740
N8	1377	2740
N9	-	2740
N10	-	2740
N11	6742	2520
N12	15048	2520
N13	8894	2520
N14	19018	2520
N15	6490	2520
N16	8490	2360
N17	13075	2360
N18	17910	2360
N19	-	2360
N20	11803	2360
N21	9961	2050
N22	3944	2050
N23	5940	2050
N24	11692	2050
N25	29177	2050
N26	-	1700
N27	12434	1700
N28	8593	1700
N29	5909	1700
N30	2293	1700

N31	4158	1490
N32	5468	1490
N33	4885	1490
N34	4926	1490
N35	6937	1490
N36	7904	1320
N37	30970	1320
N38	6841	1320
N39	6579	1320
N40	25959	1320
N41	11093	1100
N42	6826	1100
N43	21941	1100
N44	3854	1100
N45	24486	1100
N46	9408	950
N47	8202	950
N48	13949	950
N49	29908	950
N50	12833	950
N51	16802	740
N52	4638	740
N53	15766	740
N54	15131	740
N55	12991	740

Table 4 OTU richness of each sample across all 51 samples collected along the ~2,200 meters elevational range on Mt. Norikura.

Sample name	OTU richness	Elevation (m)	Elevational isocline
N1	46	2934	2940
N2	49	2934	2940
N3	52	2946	2940
N4	22	2941	2940
N5	32	2942	2940
N6	50	2739	2740
N7	69	2740	2740
N8	51	2738	2740
N11	127	2522	2520
N12	68	2518	2520
N13	78	2520	2520
N14	86	2521	2520
N15	76	2522	2520
N16	115	2360	2360
N17	112	2351	2360
N18	123	2337	2360
N20	102	2378	2360
N21	103	2027	2050
N22	129	2090	2050
N23	116	2051	2050
N24	107	2057	2050
N25	131	2070	2050
N27	134	1691	1700
N28	147	1696	1700
N29	72	1700	1700
N30	108	1707	1700
N31	91	1492	1490
N32	104	1487	1490
N33	106	1492	1490
N34	95	1500	1490

N35	108	1489	1490
N36	115	1319	1320
N37	87	1321	1320
N38	120	1321	1320
N39	81	1320	1320
N40	92	1318	1320
N41	113	1100	1100
N42	107	1105	1100
N43	130	1095	1100
N44	123	1103	1100
N45	149	1105	1100
N46	133	917	950
N47	135	960	950
N48	136	991	950
N49	36	990	950
N50	143	984	950
N51	105	744	740
N52	100	745	740
N53	126	744	740
N54	78	741	740
N55	109	750	740

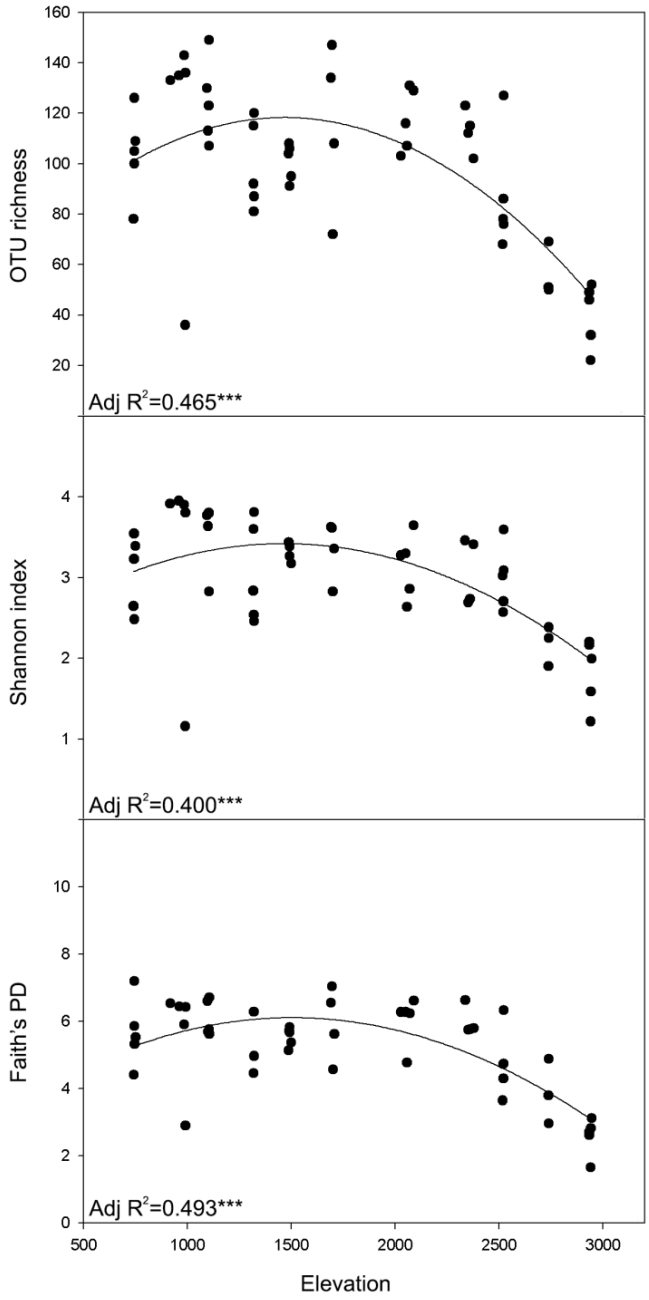
OTU richness
Adj R²=0.465***
Shannon index
Adj R²=0.400***
Faith's PD
Adj R²=0.493***
Elevation

Fig. 9 Soil nematode diversity measured along the elevational gradient. OTU richness, Shannon index, Faith's PD were used. Linear, quadratic and cubic regression models were fitted to assess the relationship of elevation with diversity indices of the nematode communities. Model selection was carried out based on adjusted R^2 and root mean square error. Only the best fit quadratic regression was shown. Significance levels were less than 0.001, shown as ***.

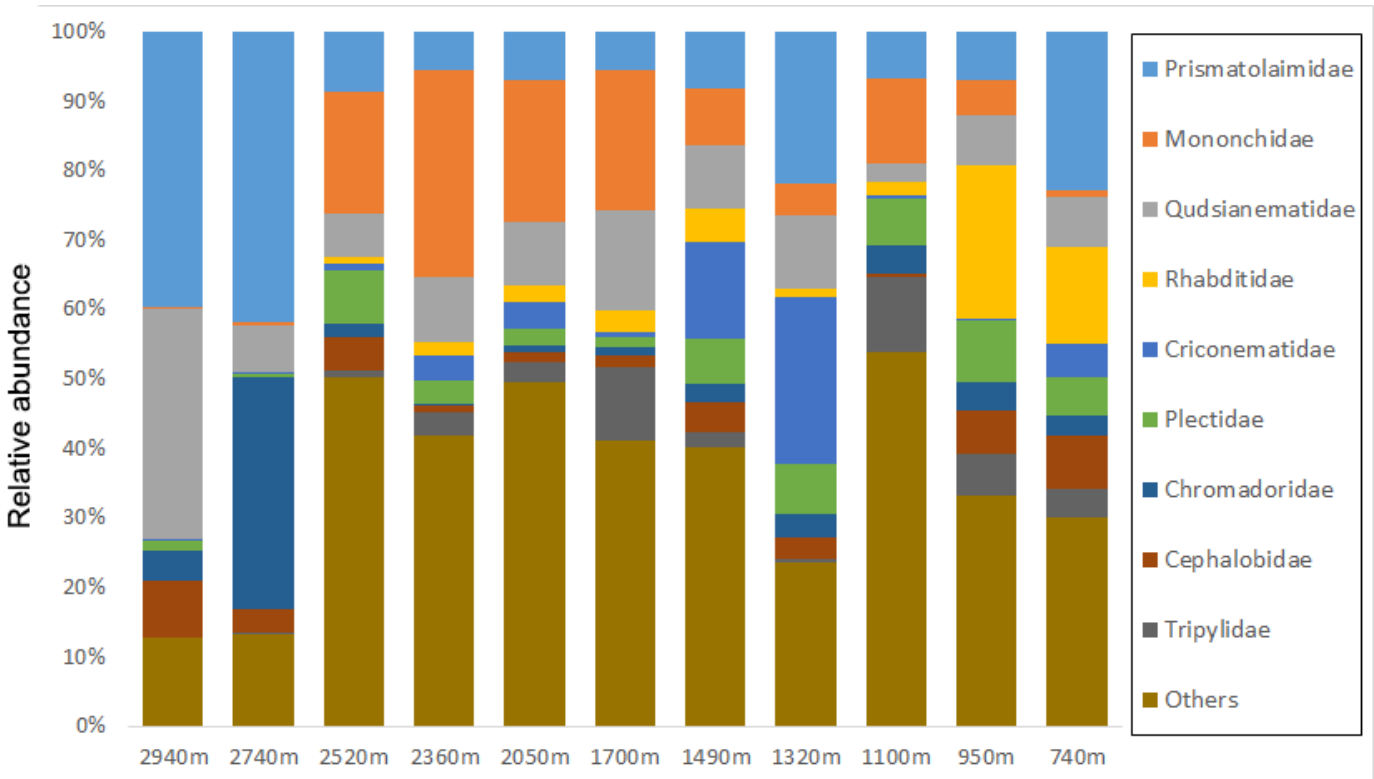

Fig. 10 Relative abundance (%) of dominant nematode families at different elevational isoclines, based on total sequence reads.

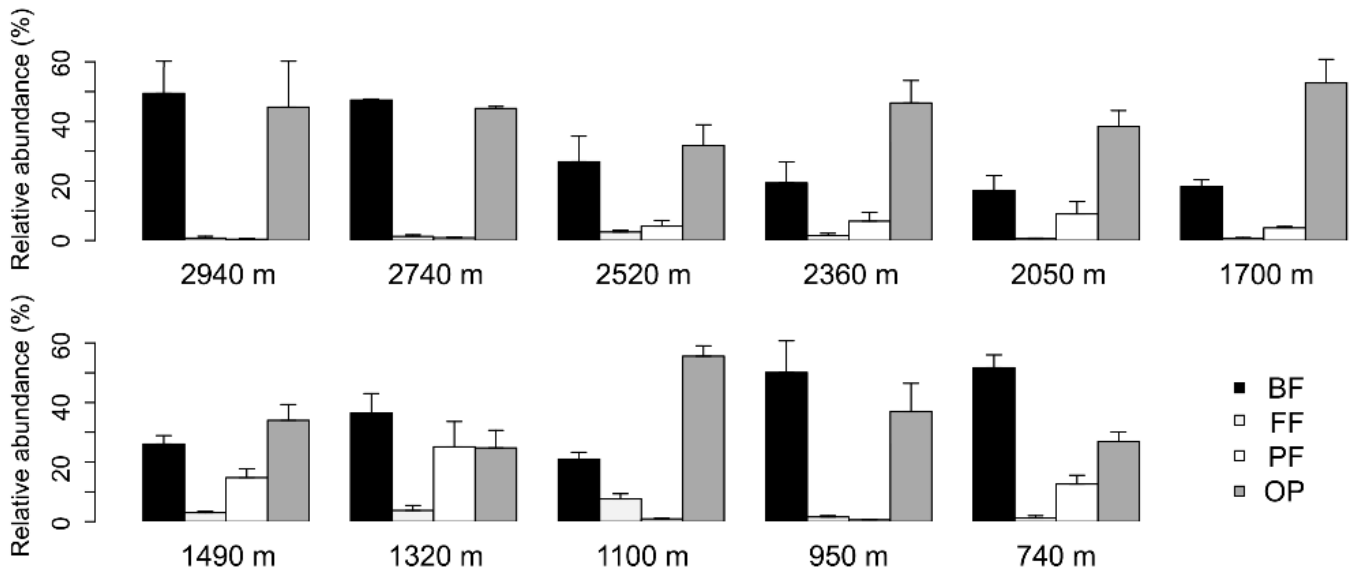

Fig. 11 Relative abundance (%) of nematode feeding guilds at eleven different elevations. BF, bacteria feeding; FF, fungi feeding; PF, plant feeding; OP, omnivore/predator.

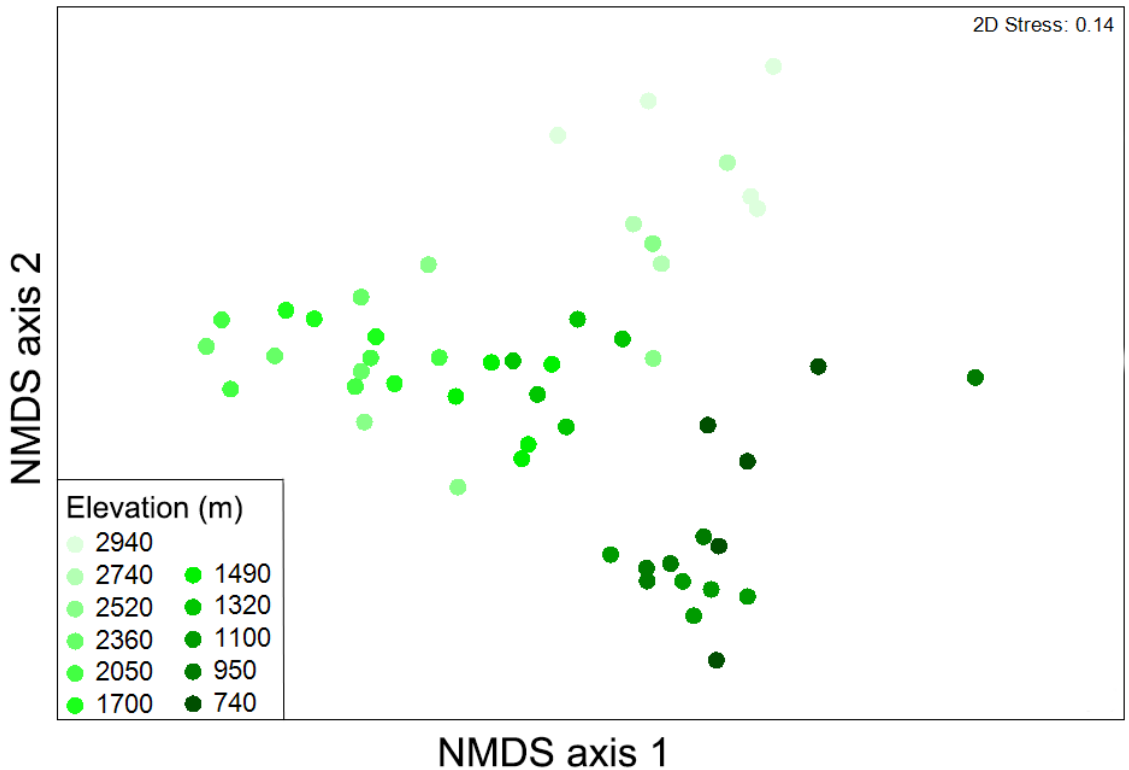

Fig. 12 NMDS of Bray Curtis similarity of overall community structure in relation to elevation.

Table 5 A packed matrix order categorizing nestedness of each sample, ordered from low to high nestedness.Samples from the elevation above 2,500 masl are highlighted.

Sample name	Elevational isocline	OTU richness
N4	2940	22
N5	2940	32
N49	950	36
N8	2740	51
N1	2940	46
N6	2740	50
N3	2940	52
N2	2940	49
N7	2740	69
N12	2520	68
N29	1700	72
N37	1320	87
N39	1320	81
N15	2520	76
N14	2520	86
N40	1320	92
N31	1490	91
N13	2520	78
N54	740	78
N34	1490	95
N20	2360	102
N32	1490	104
N35	1490	108
N33	1490	106
N21	2050	103
N36	1320	115
N38	1320	120
N42	1100	107
N23	2050	116
N30	1700	108
N18	2360	123
N52	740	100
N27	1700	134

N17	2360	112
N22	2050	129
N51	740	105
N55	740	109
N41	1100	113
N24	2050	107
N11	2520	127
N46	950	133
N47	950	135
N44	1100	123
N43	1100	130
N53	740	126
N48	950	136
N45	1100	149
N25	2050	131
N50	950	143
N28	1700	147
N16	2360	115

2.3.3. Influence of physicochemical characteristics on soil nematode community

Multiple regression analyses were used to evaluate in more detail the effect of the physicochemical characteristics on diversity indices, including OTU richness, Shannon index and Faith's PD. The multiple regression analysis showed the relative influence of physicochemical characteristics on nematode diversity indices. Seven variables were tested and TN and MAT were significantly correlated with all the

richness/diversity indices (Table 6).

On Mt. Norikura, Canonical correspondence analysis (CCA) suggested that among the physicochemical characteristics relating to elevation, pH (pseudo-$F = 3.3$, $p < 0.005$), MAT (pseudo-$F = 2.2$, $p < 0.005$), TN (pseudo-$F = 1.9$, $p < 0.005$) and C/N ratio (pseudo-$F = 1.4$, $p < 0.005$) were the significant contributors to variability of nematode communities on Mt. Norikura (Fig. 13). A total of 66.90% of variation with two axes was explained in an accumulative variance for the interaction between communities and the four significant variables.

Table 6 Linear model regression between physicochemical characteristics and diversity indices. Coefficients are shown for significant predictor variables. Significance level is shown at ***p < 0.001; **p < 0.01 and *p < 0.05. The analysis was performed after covering variables were removed and only the significant ones are shown. Abbreviations: MAT, mean annual temperature; TN, total nitrogen.

	OTUrichness (R^2=0.614***)	Shannon (R^2=0.432***)	Faith's PD (R^2=0.602***)
Intercept	57.5***	2.3***	3.57***
MAT	2.8***	0.1**	0.1***
TN	33.6***	0.6***	1.4***

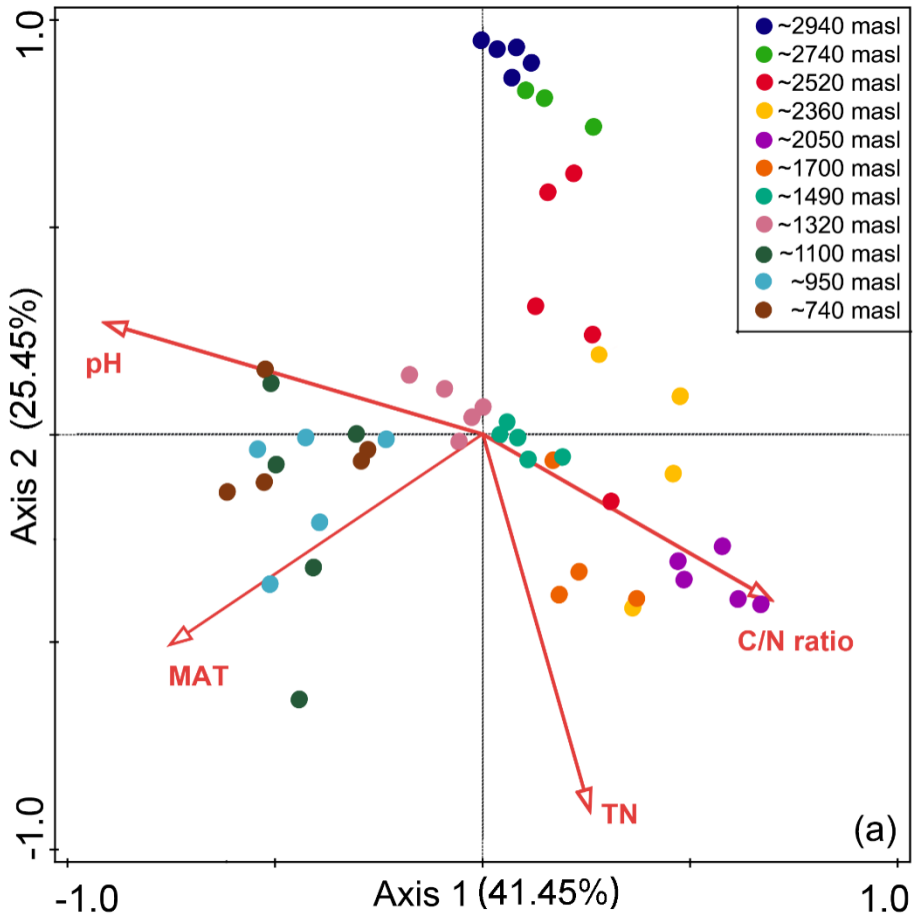

Fig. 13 Canonical Correspondence Analysis ordination plot of soil nematode community structure based on 18S gene OTUs and a vector overlay of the physicochemical characteristics. Physicochemical characteristicswere shown in arrows and only the significant ones were presented. Different colors of symbols denote different elevations.

2.3.4. Mean elevational range of nematode OTUs on Mt. Norikura

Mean elevational range of nematode OTUs was calculated, for each level on the mountain. The average species range extent for nematode community members became greater with increasing elevation ($p<0.01$) (Fig. 14).

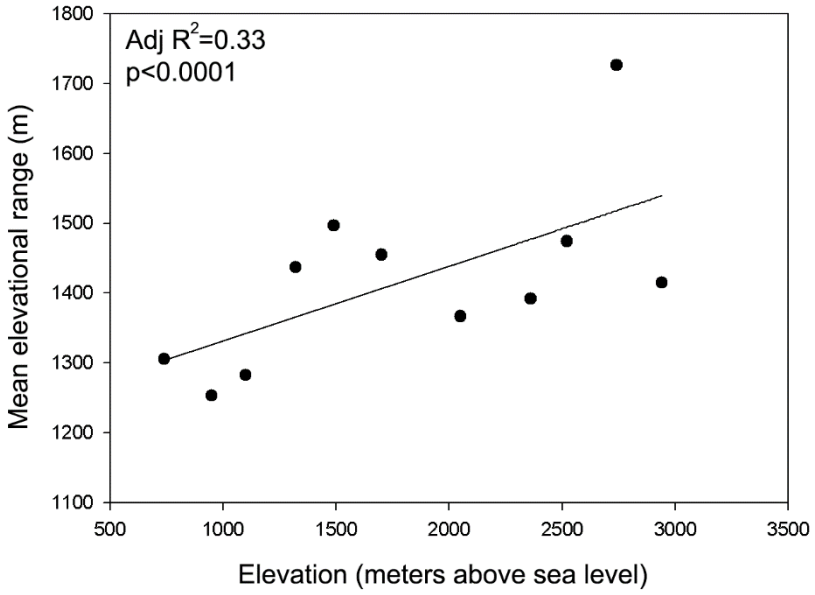

Fig. 14 The mean elevational range of high elevation nematodes have broader elevational ranges and therefore broader temperature ranges. High correlations ($p<0.01$) were found at all sequence similarities. MAT, mean annual temperature.

2.4. Discussion

In agreement with our first prediction, the diversity of soil nematodes was highly correlated with elevation, and showed a mid-elevation maximum on Mt. Norikura. Our finding adds a new example to the often-observed unimodal diversity pattern that is found along elevational gradients, although this in itself does not provide an explanation for its occurrence. There are various candidate hypotheses that could explain this pattern.

As explained in the introduction to this paper, mountains in central Japan tend to have rainfall maxima in their mid elevations (Fujimura *et al.* 1971), and it is possible that the extra soil moisture explains the survival and coexistence of more nematode species in the mid elevations. Further testing of this hypothesis would require more detailed observations both of the actual precipitation gradient on Norikura, and the effects of altering soil moisture on nematode activity and diversity.

However, the ‘community overlap’ hypothesis, which assumes that two distinct environments on a mountain each have their own distinct set of species, was obviously not effective in explaining the observed diversity pattern of soil nematode community because our results indicated that the communities of the lowermost and uppermost elevations on Mt. Norikura are not distinct. Instead, the communities of higher elevations tend to be a subset of the communities of lower elevations.

On the other hand, the midpoint attractor model, by simulation in a null model, successfully reproduced the empirical richness pattern

found on Mt. Norikura, indicating gradients of elevational favorability, subject to geometric constraints, may account for the unimodal elevational richness pattern of soil nematodes. MDE has often been put forward to explain the unimodal patterns found for various taxonomic groups along mountain elevation gradients (Singh *et al.* 2012; McCain, 2004; Brehm *et al.* 2007; Wang *et al.* 2011).

Amongst the physicochemical factors we measured, MAT and TN concentration in soil were the strongest predictors of soil nematode diversity, predicting most of the variation. In natural ecosystems, nitrogen is mostly ultimately contributed by fixation by certain bacteria and archaea, although much of the N present has re-entered the soil as plant litter (Bouwman *et al.* 1994; Griffiths, 1994).A higher soil nitrogen concentration in soil usually indicates a higher concentration of microorganisms, and this may be expected to support more abundant and diverse bacteria-feeding nematodes, and these bacteria-feeding nematodes, in turn, contribute to nitrogen mineralization by feeding on bacteria, and also by dispersing bacteria through the soil (Ferris *et al.* 1998). Generally, nitrogen concentration in soil is closely related to community diversity of soil nematodes elsewhere, and a higher nitrogen concentration tends to reflect a higher diversity of soil nematodes (Parifitt *et al.,* 2005). However, greater nitrogen concentration does not always equal greater nematode diversity. Beyond certain levels of surplus nitrogen - for example in farmland ecosystems - nematode diversity decreases (Xu *et al.,* 2007; Sarathchandra *et al.,* 2001). This is thought to be because too much organic nitrogen addition results in very high concentrations of microorganisms, leading to such dense nematode communities that

certain nematodes which are adapted of competition between nematode species reduce the overall nematode diversity in that system (Sarathchandra *et al.*, 2001).

On Mt. Norikura, the TN concentration is greatest in soils in the mid elevations (correlating with the diversity maximum), which – following global scale patterns - may result from slower decay of leaf litter products at moderately cool temperatures (Post *et al.*, 1985), resulting in accumulation of nitrogen in the upper parts of soils, which receive and accumulate partially broken down leaf litter (Post *et al.*, 1985). At lower elevations, with their increased temperatures, more rapid leaf litter decay relative to NPP tends to reduce the N concentrations in the upper soil layers [Post *et al.*, 1985]. At higher elevations above 2,500 masl, the tree canopy disappears and the vegetation opens up to scrub and then montane tundra (Miyajima *et al.*, 2007). This may also be coupled with drier conditions above the main cloud layer (Fujimura *et al.*, 1971). The lower net primary productivity at the cooler and drier higher elevations, with reduced litter fall more than compensating for slower decay, would explain why TN concentration declines towards upper elevations of Norikura, and with it the diversity of soil nematodes.

Soil temperature (as MAT) has a marked effect on nematode growth and can significantly influence the composition of nematode communities (Bakonyi *et al.*, 2007). Driven by adiabatic cooling, temperature on Mt Norikura is predicted to decline fairly steadily with increasing elevation, starting from the warmest MAT about 10°C at the lowest level we sampled (Takahashi *et al.*, 2003). While predicted temperature is closely correlated to nematode diversity on Norikura,

there is no obvious reason why it should produce a mid-elevation diversity maximum.

Our second prediction was that along elevational gradients, community structure of soil nematodes would differ in relation to elevation, producing a clear succession of communities. We did indeed find that the OTU structure of nematode communities was highly variable between different elevations, displaying a clear 'progression' of community structure towards successively higher elevations, as is often found for other groups of organisms along elevational gradients (Heaney *et al.*, 2001; Miyamoto *et al.*, 2014; Patterson *et al.*, 1998; Singh *et al.*, 2014). In this study, physicochemical factors of soils and climate were highly correlated with elevation and showed significant influences on community structure of soil nematodes, suggesting that nematodes on Mt. Norikura have quite finely divided niches in relation to the physicochemical factors that vary with elevation. Consistent with previous studies, we found MAT, TN, C/N ratio and pH were all important environmental variables influencing nematode community structure (Li *et al.*, 2014; Song *et al.*, 2016; Zhao *et al.*, 2015). This suggests that indeed, nematode OTUs tend to have predictable distributions which are linked to identifiable environmental factors.

When we classified the nematodes on Mt. Norikura in terms of family-level feeding guilds, it was striking that the upper elevations had very few plant feeders, and that the OTUs with very broad elevational ranges belonged to families classified as bacterial feeders or omnivores. This trend could be partly a result of the lack of plant cover at the uppermost elevations on Norikura where an open tundra-like vegetation prevails (41). While the fungal-based energy channel is one of the main

decomposition process in soil (47), fungal feeders were consistently a minor part of the community over the whole elevational gradient on Mt. Norikura. This may suggest a faster decomposition model based on the bacterial energy channel predominates on Mt. Norikura.

Our third prediction was that mean nematode range extent will follow Rapoport's elevational rule. We calculated the mean elevational ranges of soil nematodes occurring at each 200 m sampling level on the mountain, and found that the mean elevational range for soil nematodes was greater in nematode assemblages occurring at higher elevations. However, not all studies of invertebrate range sizes on mountains support the elevational Rapoport pattern as with the nematodes in this study. For example, McCain (2013) found there is no consistent trend of increasing range size with increasing elevation in invertebrates (McCain *et al.*, 2013). The general prediction of a Rapoport pattern as applied to elevational gradients is based on the idea that there will be greater environmental variability on long and short timescales on high mountains (Stevens 1989 & 1992). Detailed climatic and microclimatic data for Norikura do not exist, but it is clear that direct heating of the sparsely vegetated upper slopes of mountains can produce very high soil temperatures during the day in summer, followed by drastic cooling at night and during winter (Miyajima *et al.*, 2007, Takahashi *et al.*, 2003). This may require much more generalized physiological niches for soil nematodes. Additionally, if primary productivity decreases with elevation, only species that have very generalized niches may be able to gain enough food to survive at viable population densities at high elevations. The same nematode species may also be able to survive further down the mountain – by virtue of their generalized feeding

niches – allowing them to have a wide elevational range.

There are some possible implications of our study for predicting climate change effects:

*Nematodes are specialized in relation to elevational gradient and deglaciated age, and therefore in relation to climate.*The clear differentiation of nematode community structure demonstrates how closely adjusted many OTUs are to climate, and by implication their susceptibility to climate change. The mechanism by which climate controls nematode distributions may be direct (e.g. temperature effects on nematode physiology), or indirect through plant productivity and microbial activity.

On Mountain Norikura, some nematode OTUs clearly have elevational ranges of at least several hundred meters, spanning several degrees Celsius in MAT. Thus, a typical warming scenario of 2-3 ˚C over the next century (Cox *et al.*, 2000) would not be predicted to exceed the tolerance limits of most species. Based upon the degree of OTU overlap between adjacent sampling bands 200 m apart in elevation, and based on estimated lapse rate, it appears that a 1-2 ˚C warming of climate would result in a turnover of about 50% of nematode OTUs. This is may be an overestimate of turnover due to some OTUs that are actually present at both elevations being missed in one sampling and not the other, but it offers a preliminary projection that turnover in nematode communities from climate warming on mountains may be substantial.

However, there are signs of major differences in susceptibility to climate warming between different OTUs/species of nematodes. While many species are only present in our samples over a range of 2-3 ˚C or

less, there are others whose ranges extend over more than 9 ˚C - from the lowest to the highest elevations that we sampled. In the context of global climate change, this is an interesting finding. There is a ‘stable core’ of climatically very widespread nematode species might be able to survive climate change *in situ*, and remain part of a new set of nematode communities in a warmer climate. How important these species are to the functionality of the soil ecosystem is unknown, but it possible that such climatically widespread species would play an important part in the resilience of ecosystems against climate change. Most of these ‘stable core’ nematodes are in families classified as bacterial feeders and omnivores – in itself suggesting an important role in soil ecosystem functioning, for example the re-mineralization of bacterial biomass in the soil. Metagenome studiessuggest that around 98% of living biomass in the soil (excluding roots) is bacterial (Fierer *et al.*, 2012), and nematodes that feed off this mass may be key in returning nutrients such as nitrogen to forms in which plants can utilize it in new growth (Yeates, 1987; Bongers *et al.*, 1999).

However, it is also possible that these widespread nematode OTUs, even if classified as a single unit, in fact exist as different ecotypes at different climate zones on the mountain. If strong ecotypic differentiation exists, each ecotype might not be viable when climate changes around them – necessitating in-migration of other ecotypes adapted to warmer climates.

If and when climate change occurs, the adjustment of nematode communities will require in-migration of species from lower down the mountain that are able to exploit the warmer climate.

The potential migration rates of small soil animals such as

nematodes are unknown, and requires further investigation in detailed studies. However, on a mountain the amount of climate change forecast over the coming decades – for example 3˚Cby 2100 - (Cox *et al.*, 2000) translates into an equivalent shift of climate zones of only a few hundred meters at most (500 m in the case of 3 ˚C rise). Thus it is at least plausible that on the time scale of centuries, migration rates of nematodes in mountain regions might be able to keep up with the rate of climate change.

Our finding that nematode communities cluster strongly by climate zone also suggests by extension that such climate-driven community structure gradients are likely to exist horizontally across landscapes on the broader geographical scale – for example forming latitudinal series of nematode OTUs and communities analogous to those that exist for plants, birds and mammals (Grytnes *et al.*, 2003; McCain *et al*., 2004; Rahber, 2005). A single degrees Celsius rise in temperature in the mid latitude lowlands results in a 147 km shift in climate zones, requiring much greater feats of migration. The fact that such subtle climate differentiation in nematodes is likely to exist raises the possibility of widespread disequilibrium in nematode communities, with lag effects over decades, centuries or even millennia – analogous to those that have been found for trees in Europe following the rapid end of the last glacial (Svenning *et al.*, 2004).

Higher elevation nematodes are less specialized in terms of climate, and thus more resilient to climate change. There is a clear trend in average elevational range size towards the summit of Mt. Norikura, with the nematode OTUs that prevail at the upper elevational levels having ranges of around 9 ˚C on average, as compared to around 6 ˚C

for the lower elevations. It appears that the colder climate communities at upper elevations may overall be less susceptible to climate warming, because many of the species present have broad climate ranges that could easily accommodate several degrees of warming. Thus in this sense, even though they are less diverse and in a relatively 'extreme' environment, these communities may paradoxically be more stable in relation to warming than those in warmer climates at lower elevation.

It would be interesting to know how widespread the trend in climate range observed on Norikura is on other mountain systems around the world, and whether there is also a latitudinal trend in nematode ranges (following the classic Rapoport pattern, [Stevens, 1989]). If this trend is also true for lowlands generally, it may indicate that despite the very large amount of warming projected for high latitudes (Meehl, 2007), many Arctic nematode communities may largely be able to persist through it.

The apparently 'derived' nested nature of the high elevation nematode communities on Norikura adds a further dimension: the possibility that in evolutionary and ecological time nematode communities of cold climates may tend to be composed up of a subset of species that are able to extend their ranges from warmer climates. It would be interesting to compare other climate gradients – including broad lowland climate gradients – to determine whether this is more generally true.

CHAPTER 3
Total nitrogen concentration is the best predictor for community diversity of soil nematodes in the glacier retreating region of Midre Lovénbreen

3.1. Introduction

3.1.1. Overview of studies of meiofauna on Polar Regions

One of the central aims of ecology is to understand the patterns in community composition and diversity of living organisms in nature, and from these to understand the still poorly-understood mechanisms which underlie them. The polar environments of northern Svalbard, close to the KOPRI Dasan Ny-Alesun Base, offer a view of life close to its limits. One of the greatest challenges in understanding polar ecosystems is to understand how the patterns of diversity and community composition of organisms adjust themselves to local microclimates, and differences in the availability of resources. As well as giving better understanding of the survival and coexistence of different forms of life, this may also help explain the seasonal and spatial patterns of biogeochemical processes of the tundra. Also of great interest in ecology is the broader scale question of how the extreme climate and limited resources of the polar environment alter community structure, niche width and diversity in comparison to other ecosystems in warmer climates. Through such comparisons, general theories of community structure and species coexistence may be arrived at.

Over the years, there has been a considerable amount of study of plants and larger animals (from large mammals, down to insect size) in polar environments, including Svalbard (Alsos *et al.*, 2012). In contrast, very little attention has been given to local and regional diversity trends in small soil metazoa (roughly <0.1-2mm in length) in the high Arctic and Antarctic (Coulson, 2013) (often now referred to as soil ‘meiofauna’, a term borrowed from marine ecology), even though these

may be a significant part of the diversity, a large part of the biomass, and perform important functions within the ecosystem (Coulson, 2013). This is understandable because many of these organisms are very small, often only visible through a microscope or magnifying glass, and hard to distinguish from one another because of their morphological similarities.

3.1.2. Nematodes as an example of soil meiofauna

Polar nematodes appear to have even less studied than most other soil invertebrate groups (Coulson, 2013). Even though they are considered to be the most abundant animals on Earth, they have often been completely ignored in community and food web studies, even when many other small animals were included (Hodkinson *et al.*, 2004). In general, the nematode ecology of Polar Regions also appears to be the most poorly understood of any terrestrial system, as many more studies have been done at boreal, temperate and tropical latitudes in both moist climate ecosystems and deserts (Shepard *et al.*, 2002). This bias against nematodes in general, and polar nematodes in particular, is at least partly due to the practical difficulties of sampling and identifying nematodes in remote locations using traditional morphological criteria under a microscope. Of the few previous studies which have focused on tundra or polar desert nematode community distribution and diversity, the work by Kennedy (1993), Powers *et al.* (1995), Treonis *et al.* (1999) and Porazinska *et al.* (2002) in the dry valleys of Antarctica revealed a low overall diversity of nematodes (often 1-3 species in each microhabitat), but quite distinct communities adapted to particular substrates and microclimates, often separated by

just a few meters. In the polar semi-desert of Devon Island, Canada, distribution of nematodes also depended very much on microsite (Cockell *et al.* 2001). 'Micro-oases' of greater plant cover associated with nutrient concentrations, and with high populations of soil bacteria and fungi, had greater abundance and diversity of nematodes. In all of these studies, nematode diversity, while restricted to only a few species, was higher in warmer, moister and more nutrient-rich microsites. An exception is the study by Mouratov *et al.* (2001) on King George Island, just off the Antarctic Peninsula, where the sparse tundra/polar semi desert also yielded no more than 3 or 4 nematode species in most samples, but abundance and diversity was lower in the dampest microsites – perhaps because of the generally moister soil conditions at this locality, giving some waterlogged and low nutrient peaty soils.

On Svalbard, only 8 nematode genera were identified in a study by Klelowski & Opalinski (1986) on tundra at Fugelberget at the southern end of the archipelago. As in most polar studies, nematode abundance and diversity was concentrated into areas of greater moisture, vegetation cover and organic matter content. Despite their low apparent diversity, the potentially key importance of nematodes as decomposers and detritivores, and mineralizers of N and P, was recognized in the authors' description of the Svalbard tundra ecosystem. For Svalbard as a whole, 113 nematode species have been recorded through morphological identification, including both soil and shallow freshwater nematodes (Coulson, 2013), although Coulson (2013) emphasizes that on Svalbard soil invertebrates including nematodes remain relatively poorly studied.

It is unclear how the diversity and guild structure of polar

nematode communities compares with those of lower latitudes. Boag & Yeates (1998) suggested that the global peak of soil nematode diversity lies not in the tropics but between 30 and 40 degrees N or S, in the mid latitudes, and reaches its lowest point in the high latitudes above 70 degrees N and S. That conclusion was at the time based on only two studies from the Arctic, and several from Antarctica.

It is also striking that the guild structure of polar nematode communities is much simpler than in warmer climates. Studies from Antarctica's dry valleys and King George's Island reveal that plant feeders and specialised predators are mostly or entirely absent, and that the main nematode species present are bacterial feeders and omnivores (Mouratov et al. 2001).

3.1.3. Revolutionary improvement in community study

Up until recently all studies of nematodes, such as those cited above, relied on morphological criteria. However, in the past several years has it become possible to assess biodiversity of soil metazoans rapidly by bulk physical isolation of the organisms from soil and extraction of their DNA. Massively parallel sequencing of selected marker genes allows taxonomic classification and estimation of diversity and relative abundances (Porazinska *et al.* 2009). In their pioneering studies, Porazinska *et al.* (2009, 2010, 2012) demonstrated the feasibility of using bulk isolation followed by DNA extraction and 454-pyrosequencing of the small soil animal community of soils. They found that a large number of reads of both mites and nematodes were obtained, and experiments with artificial assemblages of species showed the abundance of reads reflected the biomass abundance of

each species in soil (Porazinska *et al.* 2010). When an appropriate 'weighting' factor is included to allow for likely organism size averages on a family-by-family basis, number of reads can accurately be related to numbers of individuals of each species.

Not only have these methods greatly facilitated rapid sampling, they have generally revealed a much greater 'hidden' nematode diversity than was suspected from morphological criteria. In Porazinska's studies of tropical and temperate rainforests (2009, 2012), 'species' richness defined from DNA-based methods with pyrosequencing was 2 – 3 times greater than that obtained by traditional morphology-based methods (Porazinska, pers. comm.). Most species observed in samples by this methodology were new to science, not matching the DNA profiles of any known species when tested against the 'bar codes' of morphologically described species. This was especially true of tropical rainforest. One result of this altered picture is that it now appears that nematodes may in fact show the 'typical' latitudinal diversity gradient, with a greater diversity in tropical rainforests compared to temperate forests (Porazinska *et al.* 2012). Whether this gradient continues to the high arctic latitudes is unknown, as no metagenetic studies of nematodes have yet been conducted. In general, then, it appears that nematode communities are rife with 'cryptic' species which had never before been distinguished using morphological criteria alone.

The metagenetic studies of Porazinska *et al.* (2012) showed that around half of all tropical and temperate soil nematodes are bacterial/fungal feeders, with plant parasites and predators also being predominant. This differs from the picture from classical morphological

criteria (Mouratov et al, see above). There is a need to reinvestigate the guild structure of polar nematodes using rigorous and standardised metagenetic criteria, which can give a more conclusive answer.

3.1.4. Nematode community in relation to successional gradient

Ecological succession is a gradual process of change in the composition and function of communities, which consists of non-seasonal, directional and relatively continuous patterns of species colonization and extinction (Odum, 1971, Odum, 1992, Begon *et al.*, 1990, Miles *et al.*, 1993; Miller, 1994). This process can be separated into a series of phases or stages. Early or pioneer stages tend to be stochastic as opportunistic species colonize; later stages tend to be more self-organized. Primary succession begins in landforms that have not previously been influenced by a community. Secondary succession starts in areas that have been partially or completely devoid of vegetation but where soil, seeds and spores partially remained.

Recently deglaciated areas offer striking examples of primary succession on barren grounds. Glacier forelands have been well studied with respect to soil formation and plant succession, as reviewed by Matthews (1992). Since the classic study by Crocker and Major (1995) on soil development at Glacier Bay, Alaska, considerable relevant information has been gathered from a variety of glacier forelands (Frenot *et al.*, 1995; Erschbamer *et al.*, 1999). However, there is a conspicuous lack of knowledge about soil nematode in these initial soils and its possible role in soil formation. Nevertheless, the recent

study by Lei *et al.* (2015) revealed different nematode communities showed contrasting responses to chronosequence stages on an alpine glacier foreland and showed declining characteristics in late mature phases, although no distinct retrogression (Lei *et al.*, 2015).

3.1.5. Questions and hypothesis

1) How does soil nematode diversity change during succession? We predict that soil nematode diversity and abundance will increase with ongoing succession, as primary productivity increases, allowing more levels in the food chain, and more specialized niches. As well as alpha-diversity, beta-diversity between the samples is predicted to increase. A greater number of plant species will produce a more heterogeneous environment (as shown in vertebrate studies in different systems, Pianka, 1973), allowing more soil nematode species to coexist.

With increasing ages since the deglaciation of the soil on the Midtre Lovénbreen Glacier foreland, characteristics are changing gradually. For example, the complexity of invertebrate in glacier foreland is simply continuously accumulating – with the progressive addition and persistence of taxa, and little or no loss of species over time (Vater *et al.*, 2013), while vegetation diversity firstly increases and then declines when the ecosystem became more mature (Walker *et al.*, 2003). However, no matter the diversity increases or decreases, the shifting of it happens continuously.

We are also interested in a more general way to understand which environmental variables are contributing to whatever diversity trend exists, since nematode communities are sensitive to the surrounding

environment and several environmental variables (e.g. salinity, temperature, pH, soil moisture and organic nutrient) have been identified as important factors influencing diversity of nematode communities (Freckman *et al.*, 1997; Powers *et al.*, 1998).

2) Along primary successional gradients, community structure of soil nematodes will differ in relation to the gradient, producing a clear succession of communities. Although there is very few studies has been done of soil nematode community in relation to primary succession on glacier foreland region, a recent study by Lei *et al.* (2015) revealed that different nematode communities showed contrasting responses to chronosequence stages on an alpine glacier foreland and showed declining characteristics in late mature phases.

Given that the turnover pattern in species and communities is so widespread, we hypothesized that soil nematodes, too, would have finely adapted environmental niches in relation to particular soil stages, and this would produce a series of distinctive communities along the primary successional gradient.

3) How does trophic guild structure of soil nematode change during succession? We predict that the trophic guild structure of soil nematodes will vary during succession, with more generalized bacterial and fungal feeders and omnivores of the early stages, while in later stages plant root feeders and carnivores become relatively more common.

4) To what extent are there predictable soil nematode communities and defined niches during succession? We predict that there will be a repeated and predictable series of communities associated with each stage of succession, dominated by different sets of species that are

associated with the soil conditions and levels of primary productivity. This will reveal the extent to which soil nematode species occupy distinct niches from one another during the successional process, a key to understanding the structuring of niches in soil meiofaunal communities.

3.2. Materials and methods

3.2.1. Sampling and soil analysis

The sampling sites were the foreland of the retreating Midtre Lovénbreen Glacier, Svalbard (Table 7). The glacier began to retreat in the 1920s, leaving an area of moraine which now covers around 10 km^2. Progressive time stages of the retreat have been mapped based on high resolution aerial photography and satellite imagery (Moreau *et al.*2005). Interpolation between these isochrones allows estimation of the age since deglaciation of each point in the foreland. Moreau *et al*. (2005) described the vegetation in this deglaciated area as strongly influenced by age since deglaciation, and further from successional equilibrium than tundra outside the glacial foreland zone. In the foreland, pioneer species including *Salix Polaris* are found as a sparse vegetation in younger areas, while other species such as *Carex nardina* and *Polygonum viviparum* also occur in the older foreland soils.

We took three transects, running from the current snout of the glacier to the edge of the moraine that has formed since the 1920s (Fig. 15-17). In total, 39 samples were collected from three transects from the glacier front to the edge of the foreland moraine to ensure a representative coverage of the foreland (15 samples were taken from

transect 1, 15 from transect 2, and 9 from transect 3). Because of the differing distances covered during retreat in different parts of the foreland, Transect 1 was sampled every 50 meters, and the Transect 2 was sampled every 80 meters, reflecting the longer length of the middle part of the foreland due to more rapid glacial recession. Transect 3 was sampled near transect 2 to ensure that enough samples were collected from each soil stage (Fig. 18). At each sampling site, five subsamples were taken within a $1m^2$ quadrat, taking a scoop of 100 g of soil at 0-10 cm depth at each corner and at the central point of the square. The five subsamples were pooled together to make one sample of 500g for that $1m^2$ quadrat. Sampling was carried out over 3 days at the end of July and beginning of August 2014.

Nematodes were collected by a modified Baermann funnel method, with nematodes travelling downwards through waterlogged soil and being collected in a tube below (Van Bezooijen, 2006). Immediately on arrival of the soil sample at the laboratory, 200 g of soil was wrapped loosely with 2 mm medical gauze and placed into a funnel, which was then filled with distilled water at 20 ˚C, with the apparatus kept in an air conditioned room at 20 ˚C during extraction. After 24 hours, 30 ml water in the bottom of the funnel, containing both, nematodes and soil particles was collected to centrifuge and the sediment was used to extract nematode DNA.

The Baermann funnel is used for extraction of active nematodes from plant material and soil. The sample size depends on the funnel diameter and the type of material. If extraction is from soil, the final suspension is dirty. The method makes use of nematode mobility. If material is placed in water, nematodes crawl out of the material and

sink. The method is simple and cheap and when extracting nematodes from plant material, the final suspension is virtually clean. The extraction efficiency is rather high for small samples, but for larger samples efficiency decreases exponentially. Many nematodes die due to accumulation of metabolic coreucts and microorganisms, and lack of oxygen at the bottom of the funnel. To inhibit bacterial growth add a few ml of methyl-p-hydroxybenzoate (0.15%) to the water and to avoid lack of oxygen use a 0.15% solution of hydrogen peroxide instead of water (Baermann, 1917).

Soil texture, TOC, TN, total phosphorus (TP), and pH were measured at National Instrumentation Center for Environmental Management, Korea by following the standard protocol of the Soil Science Society of America (SSSA). Moreau*et al.* (2005) combined the high resolution air photos and satellites images (1/50000) from Norwegian Polar Institute, and generated a map of successive Midre Love´nbreen glacier terminus. A linear equation was built to reflect the relationship between the soil age (years since deglaciated and exposed to open environment) and the distance (the shortest distance to the edge of glacier tongue). The age of soil of each sampling site was obtained using the distance data as a variable.

Table 7 The sites sampled in this study. GPS coordinate was used to label the sampling site of each sample. The deglaciated age of each site is interpreted by isochrones.

Sample name	N	W	Deglaciated age
KOPRI 303	78° 53′ 47″	12° 02′ 51″	0
KOPRI 6	78° 54′ 20″	12° 04′ 36″	83
KOPRI 20	78° 54′ 18″	12° 04′ 45″	81
KOPRI 47	78° 54′ 14″	12° 04′ 41″	74
KOPRI 62	78° 54′ 12″	12° 04′ 24″	70
KOPRI 103	78° 54′ 08″	12° 04′ 16″	64
KOPRI 120	78° 54′ 06″	12° 04′ 22″	61
KOPRI 132	78° 54′ 05″	12° 04′ 26″	59
KOPRI 191	78° 53′ 58″	12° 03′ 53″	46
GFL1_1	78° 53′ 32″	12° 04′ 29″	0
GFL 1_2	78° 53′ 34″	12° 04′ 37″	14
GFL 1_3	78° 53′ 35″	12° 04′ 43″	19
GFL 1_4	78° 53′ 36″	12° 04′ 52″	23
GFL 1_5	78° 53′ 37″	12° 04′ 56″	27
GFL 1_6	78° 53′ 38″	12° 04′ 59″	31
GFL 1_7	78° 53′ 40″	12° 04′ 59″	35
GFL 1_8	78° 53′ 41″	12° 04′ 60″	37
GFL 1_9	78° 53′ 44″	12° 05′ 01″	39
GFL 1_10	78° 53′ 45″	12° 05′ 10″	44
GFL 1_11	78° 53′ 46″	12° 05′ 20″	49
GFL 1_12	78° 53′ 48″	12° 05′ 27″	54
GFL 1_13	78° 53′ 50″	12° 05′ 40″	62
GFL 1_14	78° 53′ 52″	12° 05′ 45″	66
GFL 1_15	78° 53′ 54″	12° 05′ 57″	67
GFL 2_1	78° 53′ 46″	12° 03′ 34″	0
GFL 2_2	78° 53′ 49″	12° 03′ 41″	20
GFL 2_3	78° 53′ 52″	12° 03′ 49″	25
GFL 2_4	78° 53′ 55″	12° 03′ 53″	36

GFL 2_5	78° 53′ 58″	12° 03′ 59″	47
GFL 2_6	78° 54′ 00″	12° 04′ 11″	52
GFL 2_7	78° 54′ 03″	12° 04′ 20″	57
GFL 2_8	78° 54′ 07″	12° 04′ 23″	62
GFL 2_9	78° 54′ 10″	12° 04′ 25″	67
GFL 2_10	78° 54′ 14″	12° 04′ 30″	72
GFL 2_11	78° 54′ 17″	12° 04′ 35″	78
GFL 2_12	78° 54′ 20″	12° 04′ 17″	82
GFL 2_13	78° 54′ 20″	12° 04′ 33″	83
GFL 2_14	78° 54′ 22″	12° 04′ 25″	85
GFL 2_15	78° 54′ 24″	12° 04′ 31″	87

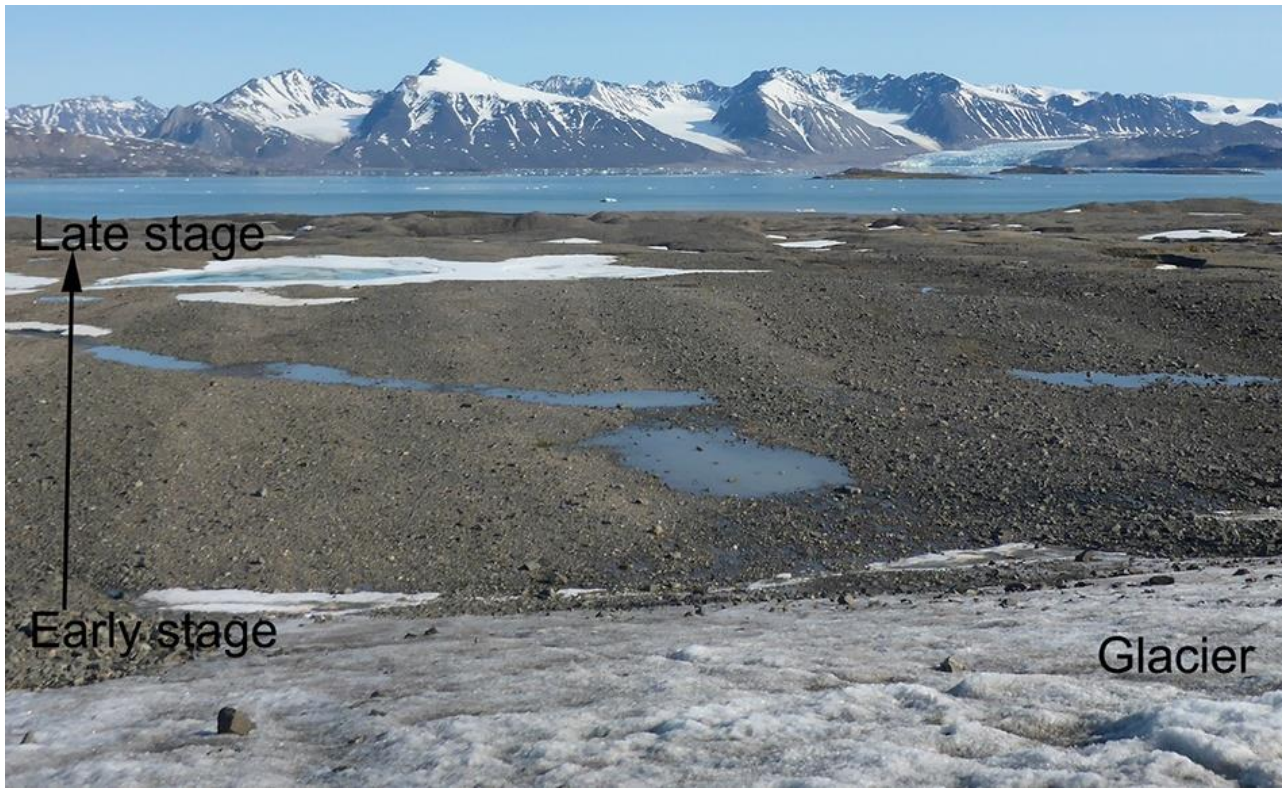

Fig. 15 Photos ofsampling sites in Midre Lovénbreen glacier showing soil stages.

Fig. 16 Photos ofsampling sites in Midre Lovénbreen glacier showing three sampling transects.

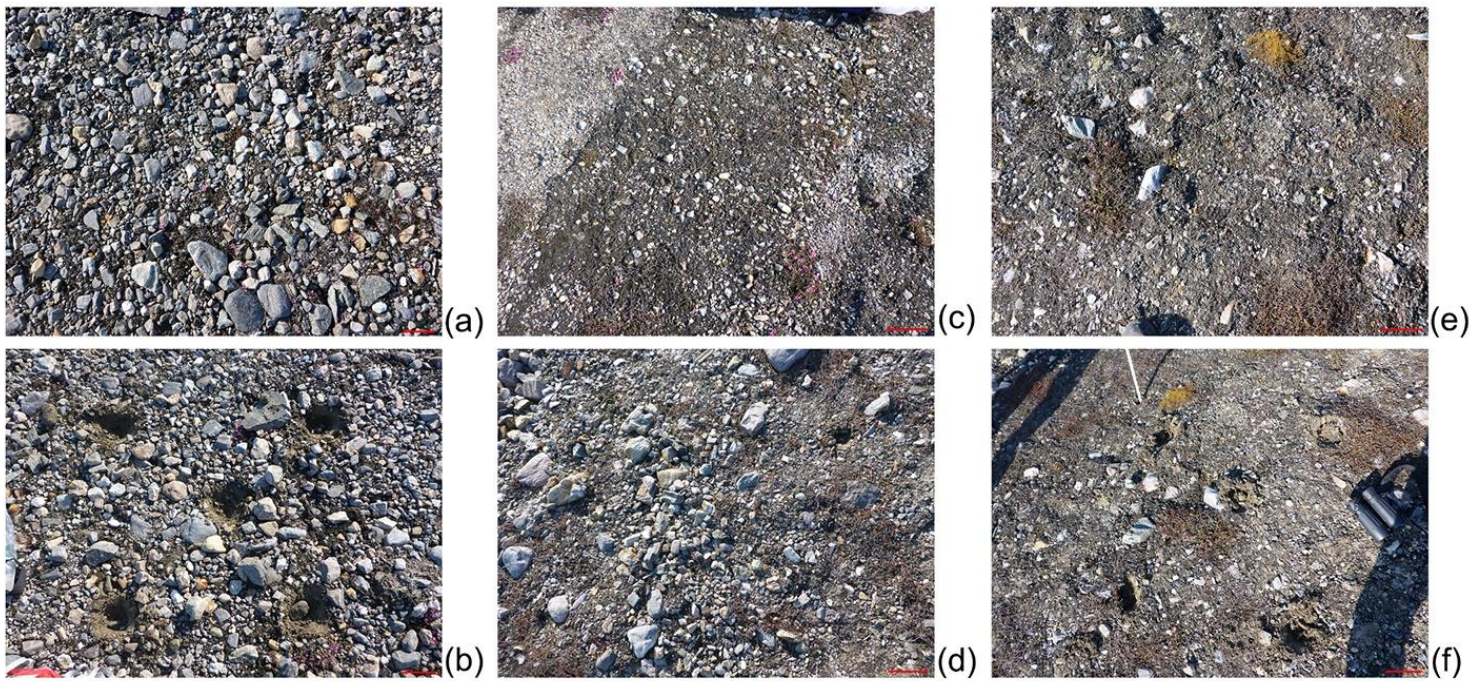

Fig. 17 Photos of sampling sites in Midre Lovénbreen glacier showing soils of three stages. (a)(b), early stage; (c)(d), mid stage; (e)(f), late stage.

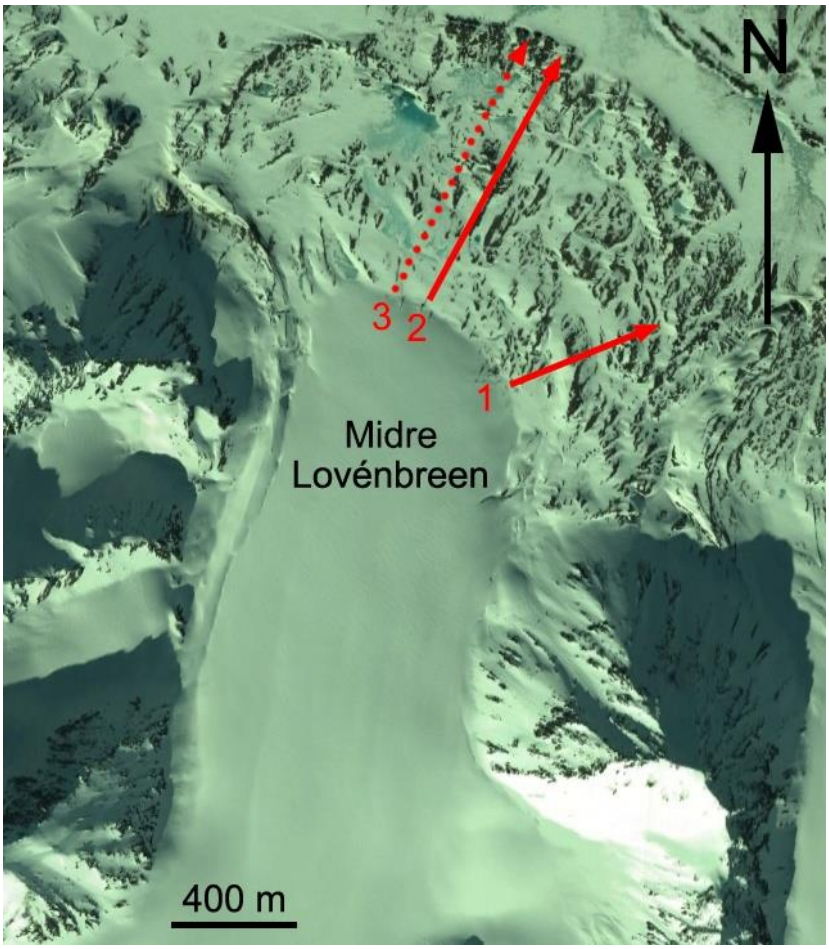

Fig. 18 Map of successive Midre Lovénbreen glacier terminus obtained from 1/50,000-scale aerial photographs (Moreau, 2005).

3.2.2. DNA extraction, amplification, sequencing and quality control of 18S rRNA gene

DNA was extracted using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer's instructions, and random empty vials were chosen and run to serve as controls. The isolated DNA was stored at -80 ˚C until the PCR stage.

Miseq sequencing was used to assess the nematode community through massively parallel sequencing of the 18S rRNA gene. Compared to the previous studies conducted using 454 sequencing platform (Porazinska *et. al.*2009), this generated a larger data volume, which could minimize sampling errors. A 350-bp region of the 18S small-subunit rRNA gene was amplified with the primer pairs NF1 and 18Sr2b (Porazinska *et. al.*2009) with adapter sequences for the Illumina MiSeq. PCR was performed in a 50-µl reaction mixture composed of 1 or 2 µl of DNA extract, 0.4 µM of each primer, 0.2mM of each dNTP mix, 1X Taq Reaction Buffer and 1.25 U Solg™ Taq DNA Polymerase (SolGent co., Ltd., Korea). PCR conditions were 10 min at 95°C, followed by 30 cycles of 60 s at 95°C, 45 s at 50°C, and 180 s at 72°C. Final elongation was at 72°C for 10 min using the C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Index PCR was performed for the purified PCR products using the Nextera XT Index kit (Illumina, Inc., San Diego, CA, USA). Each of the 50-µl reaction mixtures was composed of 5 µl of each index primer, 2× PCR Solution Premix Taq™ DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), and 5 µl of the purified DNA. PCR conditions were 3 min at 95°C, followed by 10 cycles of 30 s at 95°C, 30 s at 55°C,

and 30 s at 72°C. Final elongation was at 72°C for 5 min.

After purification by the AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA), each amplicon was normalized to 4 nM with 10 mM Tris–HCl (pH = 8.5) and pooled with an internal control PhiX (30%). Heat-denatured pooled amplicons were loaded to a v3 600 cycle-kit reagent cartridge (Illumina, Inc.), and 2 × 300 bp paired-end sequencing was performed by the Illumina MiSeq at the Graduate School of Public Health, Seoul National University.

The sequences were demultiplexed and trimmed with a read quality score above 20 by the MiSeq Reporter v2.5 (Illumina, Inc.). The sequence data obtained was processed following the Miseq SOP in Mothur (Schloss *et al.* 2009). Sequences with any ambiguous bases, sequences with more than 8 homopolymers and sequences with lengths less than 200 bp were removed using the screen.seqs command in Mothur. Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within Mothur in de novo mode. Rare sequences (less than 10 reads) were removed to avoid the risk of including spurious reads generated by sequencing errors (Brown *et al.* 2015).

The analysis of nematode richness/diversity and community structure of soil nematodes was conducted separately three times, respectively setting the cutoff points of OTUs at 99%, 98% and 97% sequence similarity. These different sequence similarity levels were used to study the possible impacts of methodological biases in OTU definition, as the average phylogenetic distance between different nematode species is unclear (Brown *et al.* 2015). At different sequence similarity level, different subsampling depths were applied according to

the lowest read number generated by sequencing of samples. Samples with too low read numbers were removed from further analysis.

Taxonomic classification of each OTU was obtained by classifying alignments against SILVA Release 115 databases (Quast *et al.* 2013) using the classify command in Mothur at 80% cutoff with 1000 iterations. The Miseq sequence data used in this study are deposited in the MG-RAST server (Meyer *et al.*, 2008).

3.2.3. Statistical analysis

Diversity indices such as Shannon index and Faith's PD and OTU richness were calculated using the Mothur platform (Schloss *et al.* 2009). To assess the best fitting model of correlations between elevation and richness/diversity and environmental variables, linear and polynomial (quadratic) models were tried out using SigmaPlot v 10.0 (Systat Software, San Jose, CA). Model selection was carried out based on adjusted R^2 and RMSE (root mean square error). We used ANOVA for normal data and Kruskall-Wallis tests for non-normal data to test whether physicochemical characteristics differed among different elevations.

To evaluate in more detail the effect of these environmental variables on OTU richness, Shannon Index and Faith's PD, we performed multiple regression analyses. Non-significant predictor variables were removed sequentially until only significant variables were left in the model. Before applying multiple regression to the dataset, we looked for redundant physicochemical characteristics using the Varclus procedure in the Hmisc package (SAS Institute, 1990) in the R platform. Cluster analysis of environmental variables and age

since deglaciation in the retreating glacier area showed high correlation between TOC and TN (Spearman $\rho^2 \geq 0.6$; Fig. 19), suggesting TOC and TN are covariables.

We performed a Non-metric Multidimensional Scaling (NMDS) using Primer v6 to explore patterns in community structure using a Bray Curtis similarity matrix. Abundance data of OTUs were square root transformed to calculate the distance between soil nematode communities. Community structure was compared between elevations using ANOSIM with 999 random permutations (Clarke & Gorley 2006).

To individually assess the influence of each environmental variables, canonical correspondence analysis was used in CANOCO ver. 5. Forward selection was used to select significant explanatory variables with 999 permutations and only significant ($p < 0.05$) variables were included in the models.

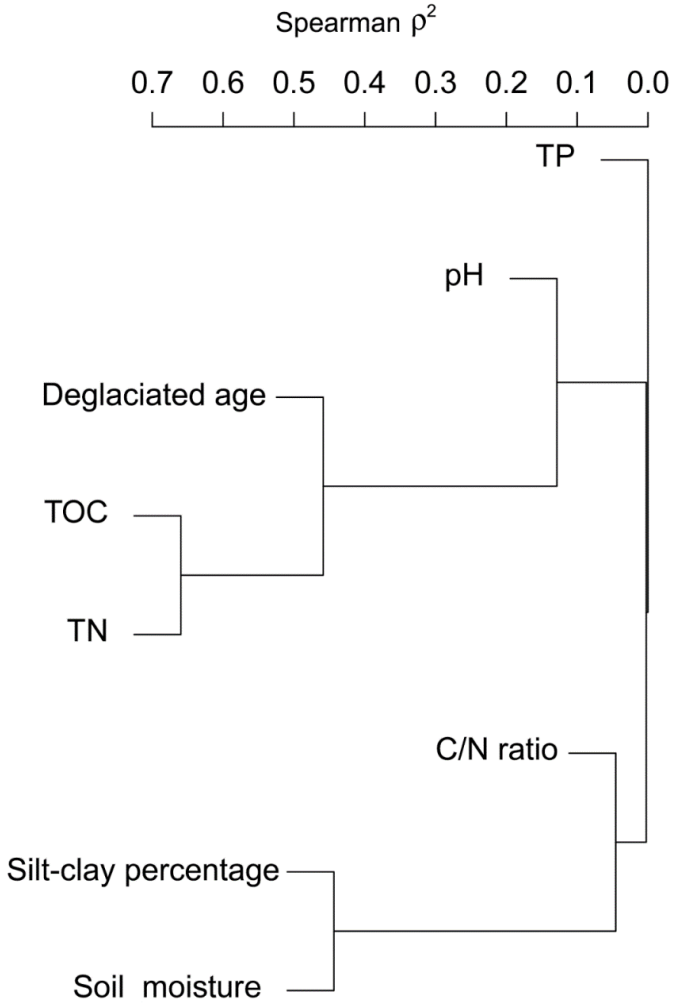

Fig. 19 Cluster analysis of all measured environmental variables and age since deglaciation in the retreating glacier area.

3.2.4. Analyzing feeding groups of nematodes

We investigated the relative abundance of the dominant nematode families across all sites. Nematodes were identified to family and binned into their respective functional feeding guilds using previous published comprehensive lists (Yeates *et al.* 1993). Four feeding groups were created: (1) bacteria feeding, (2) fungi feeding, (3) plant feeding, and (4) omnivore/predator.

3.3. Results

3.3.1. Physicochemical characteristics

With age since deglaciation, concentrations of important nutrients for microbial activity such as TOC and TN increased steadily, while pH decreased (Fig. 20). The TOC in the soil increased significantly from a very low content of 0.03% in the newly exposed soils, which are close to the edge of glacier, to over 0.69% in the oldest foreland soils around 80 years old ($R=0.66$, $p<0.0001$), while the content of TN increased from 0.004% to 0.055% ($R=0.67$, $p<0.0001$). In contrast, the pH - which ranged from 7.8 to 9.4 in the whole sample set - decreased along the chronosequence ($R=0.38$, $p<0.05$). No significant time-related patterns were found in TP, C/N ratio, soil moisture, and sand or clay-silt proportion in relation to age since deglaciation (Table 8).

Table 8 Measured soil properties of samples collected in the glacier of Midtre Lovénbreen. Soil texture were shown as the percentage of silt and clay content.

Sampling number	Soil texture	pH	Total C (%)	Total N (%)	Total P (%)	C/Nration	Soil moisture (%)	Deglaciated age
S1	36.72	9.1	0.17	0.007	442.81	24.2	11.4	1
S2	20.72	9.1	0.17	0.013	446.24	13.1	9.6	83
S3	8.16	9.4	0.15	0.014	483.72	10.7	7.8	81
S4	14.92	9.4	0.13	0.015	483.09	8.7	7.0	74
S5	4.72	9.3	0.22	0.016	657.06	13.8	9.8	70
S6	24.04	8.7	0.42	0.029	487.92	14.5	11.7	64
S7	39.64	9.0	0.39	0.03	480.1	13.0	13.8	61
S8	37.12	7.0	3.71	0.322	338.92	11.5	43.8	-
S9	46.72	8.1	1.76	0.182	456.56	9.7	25.5	-
S10	30.92	6.1	3.82	0.34	462.33	11.2	38.3	-
S11	33.16	9.1	0.24	0.022	538.52	10.9	12.8	59
S12	32.36	9.2	0.19	0.011	477.16	17.3	13.2	46
S58	11.88	9.1	0.06	0.007	527.09	8.6	2.9	0
S59	9.64	9.2	0.11	0.005	447.73	22.0	3.2	14
S60	10.68	9.0	0.1	0.005	473.65	20.0	3.3	19
S61	10.0	8.7	0.03	0.004	495.11	7.5	3.8	23
S62	9.36	8.2	0.04	0.004	434.45	10.0	8.8	27

S63	11.88	9.0	0.07	0.009	518.03	7.8	10.1	31
S64	30.72	8.4	0.03	0.018	497.92	1.7	10.3	35
S65	28.48	8.8	0.06	0.016	489.5	3.8	10.8	37
S66	12.8	8.9	0.13	0.004	480.01	32.5	8.6	39
S67	23.88	9.0	0.23	0.013	490.26	17.7	11.5	44
S68	11.16	8.0	0.23	0.015	516.17	15.3	4.2	49
S69	31.68	8.5	0.32	0.019	537.91	16.8	13.7	54
S70	23.08	8.6	0.36	0.024	588.38	15.0	11.5	62
S71	26.24	8.7	0.36	0.018	550.32	20.0	10.1	66
S72	21.84	8.3	0.5	0.034	602.71	14.7	11.3	67
S73	35.44	9.1	0.23	0.006	449.48	38.3	12.7	0
S74	24.6	9.1	0.11	0.006	471.04	18.3	12.9	20
S75	13.08	9.2	0.18	0.008	510.16	22.5	10.1	25
S76	37.8	8.7	0.28	0.013	520.4	21.5	15.5	36
S77	13.0	9.0	0.21	0.047	581.43	4.5	7.5	47
S78	24.48	8.9	0.25	0.013	581.43	19.2	13.9	52
S79	48.92	8.7	0.31	0.022	472.44	14.1	14.5	57
S80	35.56	8.5	0.34	0.027	395.3	12.6	12.7	62
S81	8.44	8.7	0.38	0.027	628.33	14.1	12.3	67
S82	8.12	8.3	0.37	0.028	546.19	13.2	10.7	72
S83	8.84	8.6	0.55	0.042	496.33	13.1	10.5	78
S84	8.48	8.0	0.69	0.06	331.02	11.5	6.4	82
S85	25.32	7.8	0.71	0.06	424.15	11.8	9.7	83
S86	9.92	8.4	0.26	0.019	435.12	13.7	7.2	85
S87	14.88	7.8	0.69	0.055	441.82	12.5	14.9	87

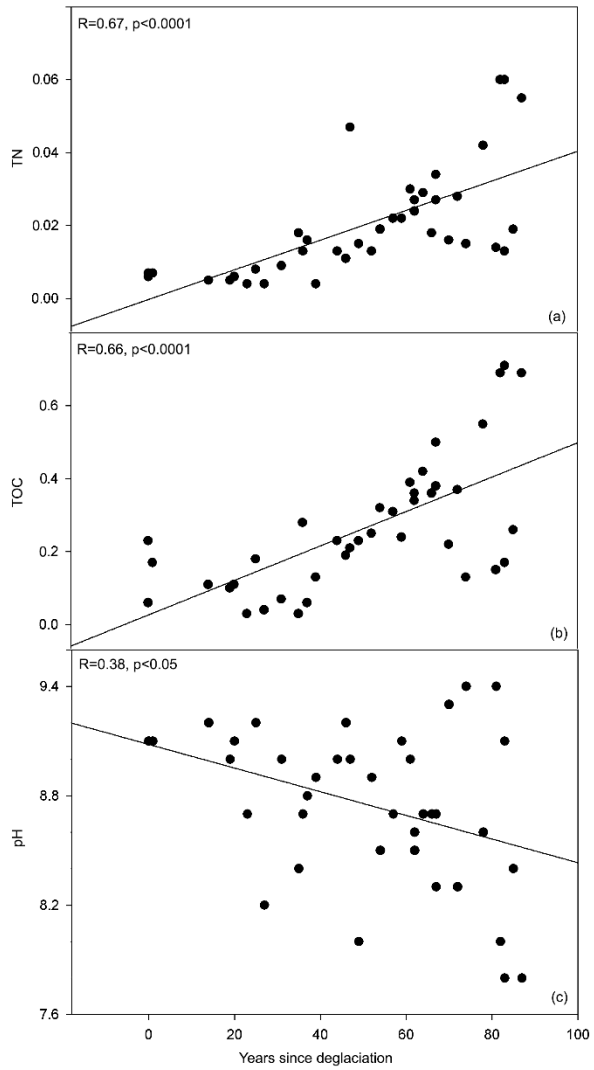

Fig. 20 Three environmental parameters are shown along the age since deglaciation of soil in the glacier of Midtre Lovénbreen: (a), TN; (b), TOC; (c), pH. TOC, total organic carbon; TN, total nitrogen. The values of TOC and TN are shown in percentages.

3.3.2. Soil nematode community

In total, 39 samples were collected from the foreland of the retreating glacier. Nine samples were excluded from further nematode community analysis owing to failure of amplification of nematode 18S rRNA genes (Table 9). Only results at 99% sequence similarity level were shown in analysis of OTUs, because different sequence similarity levels showed very similar patterns.

A total of 30,270 high quality 18S rRNA gene sequences were assigned to 1,063 operational taxonomic units (OTUs) at ≥99% similarity level from 30 samples, after the removal of low quality, chimeric, and rare sequences. On average, 56.9 OTUs (±3.6 SE) were found in each sample (Table 10). The greatest OTU richness was found at early successional stage with 103 OTUs, while the lowest OTU richness was found at late stage with 27 OTUs.

The nematode community on Midre Lovénbreen glacierwere dominated by Plectidae which accounted for 27.1% of the total reads, followed by Qudsianematidae (19.3%), Monhysteridae (9.9%), Dolichodoridae (5.9%), Criconematidae (5.4%), Tylenchidae (4.9%), Prismatolaimidae (3.8%), Mononchidae (3.3%) and Aphelenchida (3.1%). Remaining 8.0% of the total reads were shared by 9 nematode families together, and 9.3% nematode sequences remained unclassified on family level (Fig. 21).

Classifiable reads were binned into four feeding guilds (bacteria feeding guild [BF], fungi feeding guild [FF], plant feeding guild [PF] and omnivore/predator guild [OP]) according to their family taxonomy. All feeding guilds of nematode were found in the glacier retreating region (Fig. 22). BF and OP together accounted for the major portion of

73.7%, while the portion in early stage (years since deglaciation less than 60) decreased from 78.0% to 70.0% in late stage (years since deglaciation more than 60). FF were found at low abundances across all elevations of 3.3% in total.

An NMDS, performed on the Bray-Curtis similarity matrix of nematode community structure, showed the communities of early stage soil in the deglaciated region are very distinct from the communities of late stage (ANOSIM: $R=0.259$, $p<0.001$) (Fig. 23). While the communities of early stage soil were significantly different with the nematode communities of the nearby mature tundra area (ANOSIM: $R=0.63$, $p<0.01$), the communities of late stage soil were not very different with the nematode communities on the nearby mature tundra area (ANOSIM: $R=0.21$, $p>0.05$).

OTU richness, Shannon index and Faith's PD were used to assess the ecological pattern in nematode diversity. Unlike the diversity pattern found on Mt. Norikura that all of the diversity indices showed strong correlation with the gradient, there was no pattern on community diversity/richness found on Midre Lovénbreen glacier along the primary successional gradient (Fig. 24). However, the diversity indices was highly correlated to TN and pH, with increasing TN or lower pH comes higher community diversity/richness (Fig. 25).

No nested structure in term of nematode community was found. It is different as what I was expected that early stage communities would tend to be subsets of the communities from late stages.

Table 9 Nematode reads generated by Miseq sequencing of each sample and sampling depth at 99% sequence similarity level.

Sample name	Nematode reads	Deglaciated age
S1	-	1
S2	17771	83
S3	27660	81
S4	9028	74
S5	11185	70
S6	23705	64
S7	27824	61
S11	37111	59
S12	-	46
S58	-	1
S59	-	14
S60	-	19
S61	-	23
S62	12573	27
S63	1557	31
S64	31857	35
S65	28585	37
S66	2914	39
S67	15214	44
S68	16976	49
S69	20450	54
S70	18085	62
S71	15260	66
S72	19772	67
S73	-	1
S74	-	20
S75	19242	25
S76	1799	36
S77	11387	47
S78	6396	52
S79	15896	57
S80	10611	62
S81	13104	67
S82	1439	72
S83	4825	78
S84	-	82

S85	1186	83
S86	2311	85
S87	2904	87

Table 10 OTU richness of each sample across all 30 samples on Midre Lovénbreen glacier.

Sample name	OTU richness	Deglaciated age
S2	33	83
S3	37	81
S4	36	74
S5	31	70
S6	52	64
S7	39	61
S11	68	59
S62	27	27
S63	44	31
S64	52	35
S65	55	37
S66	52	39
S67	51	44
S68	79	49
S69	52	54
S70	42	62
S71	37	66
S72	75	67
S75	60	25
S76	45	36
S77	42	47
S78	50	52
S79	69	57
S80	84	62
S81	64	67
S82	80	72
S83	103	78
S85	71	83
S86	95	85
S87	83	87

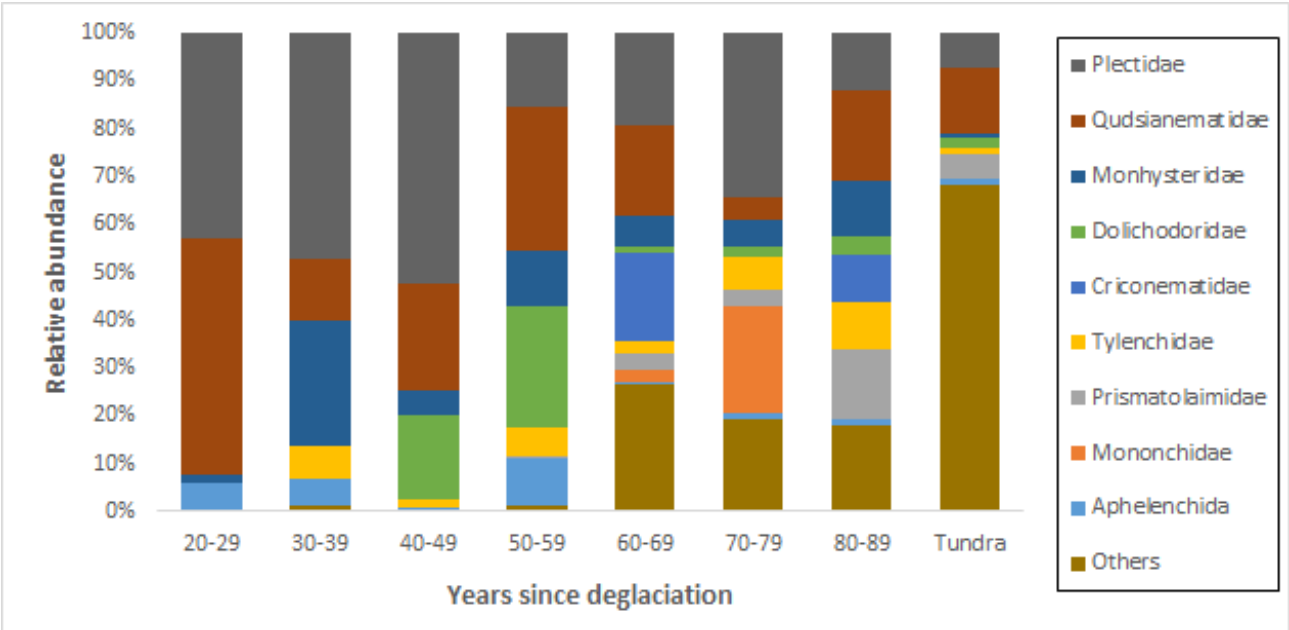

Fig. 21 Relative abundance (%) of dominant nematode families of different deglaciation isoclines, based on total sequence reads.

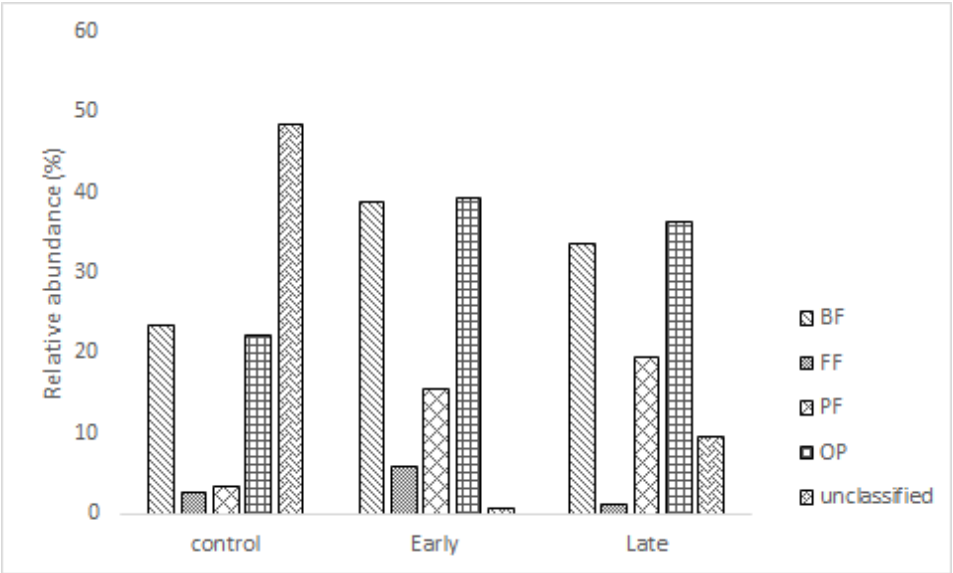

Fig. 22 Relative abundance (%) of nematode feeding guilds at three soil stages. BF, bacteria feeding; FF, fungi feeding; PF, plant feeding; OP, omnivore/predator.

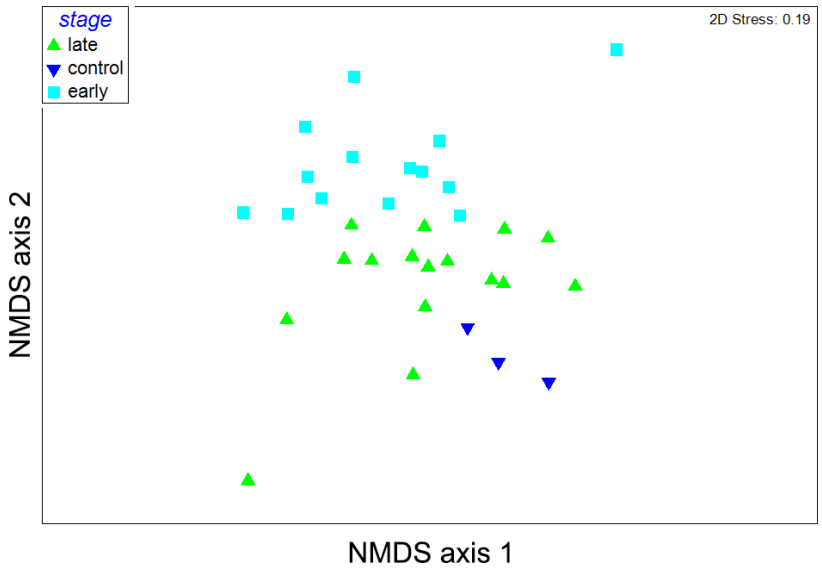

Fig. 23 NMDS of Bray Curtis similarity of overall community structure in relation to early stage community and late stage community.

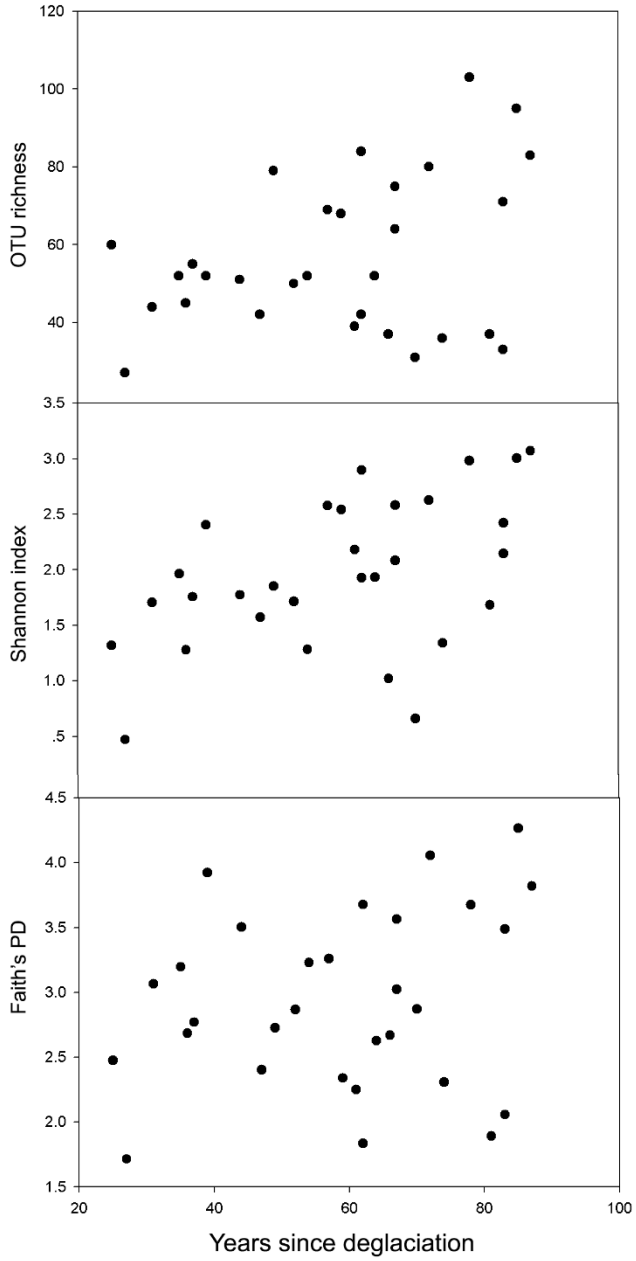

Fig. 24 Soil nematode diversity measured as OTU richness, Shannon index, Faith's PD. No trend was found.

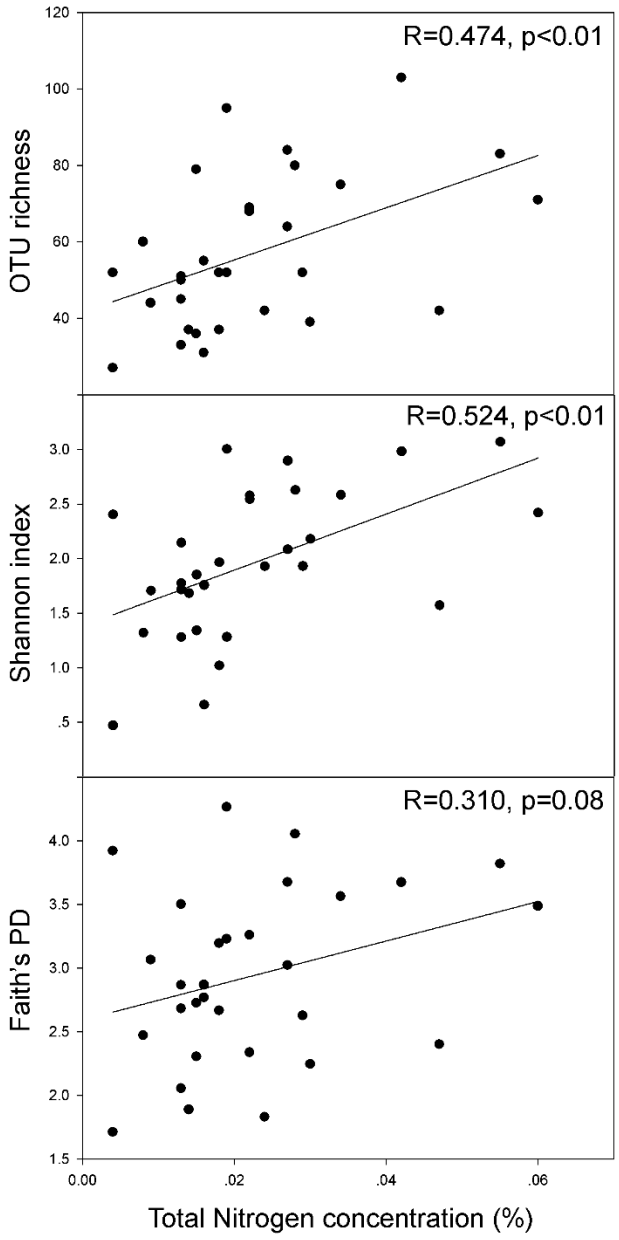

Fig. 25 Soil nematode diversity measured as OTU richness, Shannon index, Faith's PD were highly correlated to TN.

3.3.3. Influence of physicochemical characteristics on soil nematode community

Multiple regression analyses were used to evaluate in more detail the effect of the physicochemical characteristics on diversity indices, including OTU richness, Shannon index and Faith's PD. The multiple regression analysis showed the relative influence of physicochemical characteristics on nematode diversity indices. Seven variables were tested and TN and pH were significantly correlated with all the richness/diversity indices (Table 11).

On the deglaciated foreland region, CCA analysis was used to reveal which physicochemical characteristics best predicted variation in nematode communities in the deglaciated foreland area (Fig. 26). Together with two axes on the biplot, in an accumulative variance for the interaction between communities and variables, a total of 31.48% of variation was explained. Axis 1 explained 16.61% of the variation in the data, while axis 2 explained 14.87%. Axis 1 and 2 together separated out the early and late soil stages, with Late Stage soils associated with low values of CCA axis 1. TC, pH, C/N ratio, soil moisture, the proportion of silt and clay, age since deglaciation, TN and TP together accounted for 30.3% of the total nematode community variation in combination. Among these selected environmental factors, deglaciated age (pseudo-$F=1.4$, $p<0.01$) and pH (pseudo-$F=1.2$, $p<0.05$) were significant contributors to nematode community variability, and a forward test indicated that the most important factor was deglaciated age.

Table 11 Linear modelregression between physicochemical characteristics and diversity indices. Coefficients are shown for significant predictor variables. Significance level is shown at ***p < 0.001; **p < 0.01 and *p < 0.05. The analysis was performed after covering variables were removed and only the significant ones are shown. Abbreviations: TN, total nitrogen.

	OUT richness (R^2=0.280***)	Shannon (R^2=0.248***)	Faith's PD (R^2=0.180***)
Intercept	280.0***	1.4***	9.33***
pH	-25.6**	-	-0.73*
TN	-	25.6**	-

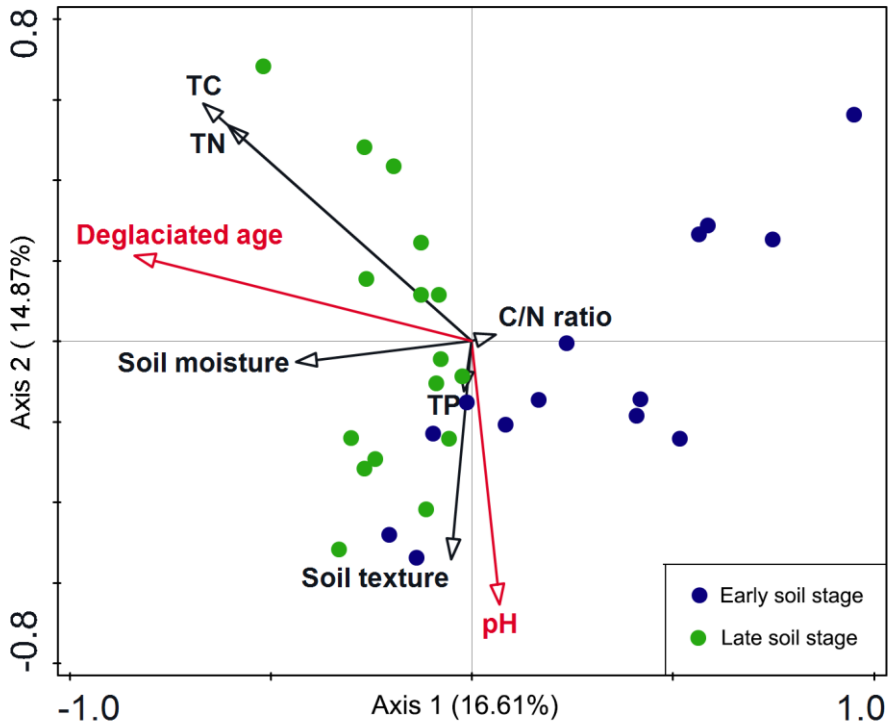

Fig. 26 Canonical Correspondence Analysis ordination plot of soil nematode community structure based on 18S gene OTUs and a vector overlay of the physicochemical characteristics. Physicochemical characteristics were shown in arrows and the significant ones were presented in red. Different colors of symbols denote different soil stage on the glacier foreland.

3.4. Discussion

Consistent with previous studies, we found TN and pH were important environmental variables influencing nematode community diversity (Li *et al.*, 2014; Song *et al.*, 2016; Zhao *et al.*, 2015). This suggests that nematode OTUs tend to have predictable distributions which are linked to identifiable physicochemical characteristics. However, no significant trend was found in diversity was found with deglaciated age on Glacier Midre Lovénbreen. Previous studies have been carried out on the glacier of Midre Lovénbreenand found that the total diversity of soil fungi increases firstly with the increasing deglaciation age and kept falling down after a maximum in diversity. Nevertheless, other studies in other deglaciated regions also have found clear trends in community diversity on bacteria and plants. The reason why there was no significant trend of the diversity of nematode and deglaciated ages could because nematodes, not like other primary colonizers, play too complicated roles to show a certain pattern in a primary succession.

Amongst the physicochemical characteristics we measured, in primary successional gradient, TN concentration in soil were strong predictor of soil nematode diversity, and pH was strong predictor of community structure of soil nematode. The most significant influences on community structure of soil nematodes suggests a large part of the difference in the nematode community structure relates to soil chemistry. The nematode communities on the deglaciated region of Glacier Midre Lovénbreen were significantly different between early stages and late stages and OTU composition of nematode communities

was highly variable across the years since deglaciation, displaying a clear ‘progression’ of community structure towards the nematode communities on tundra. This result – with clustered communities in replicates from the same and similar gradient - indicates firstly that nematode OTUs tend to have predictable distributions which are linked to identifiable environmental factors, and secondly that many of them have quite finely divided niches in relation to the environmental factors that vary with the gradients. Finding such an elevational pattern would confirm the importance of subtle niche limitations for nematode community structure. If we had found the same communities of nematodes almost everywhere, this would instead have emphasized their survival as ecological generalists, and possibly their relative insensitivity to changes in climate.

In natural ecosystems, as a result of natural nitrogen fixation by certain bacteria and archaea (Bouwman *et al.*,1994; Griffiths, 1994), a higher soil nitrogen concentration in soil indicates a higher concentration of microorganisms and therefore more abundant and diverse bacteria-feeding nematodes, and the bacteria-feeding nematodes, in turn, contribute to nitrogen mineralization by feeding on bacteria, and also by dispersing bacteria through the soil (Ferris *et al.*, 1998). Generally, nitrogen concentration in soil is closely related to community diversity of soil nematodes elsewhere, and a higher nitrogen concentration tends to reflect a higher diversity of soil nematodes (Parfitt *et al.*, 2005).However, greater nitrogen concentration does not always equal greater nematode diversity. Beyond certain levels of surplus nitrogen - for example in farmland ecosystems - nematode diversity decreases (Xu *et al.*, 2007;

Sarathchandra *et al.*, 2001). This is thought to be because too much organic nitrogen addition results in very high concentrations of microorganisms, leading to such dense nematode communities that certain nematodes which are adapted of competition between nematode species reduce the overall nematode diversity in that system (Sarathchandra *et al.*, 2001).

This study has dealt with stable end points of ecosystem development, and these may well represent the eventual outcome of change in nematode communities under climate change. However, there is the important unknown of how long it will take for nematode species to disperse to take advantage of new zones of potential climate. This will surely depend partly on the time taken for plants that form the characteristic vegetation zone to disperse, and for ecosystem development to proceed. The closeness of the relationship of nematode community structure to soil factors emphasizes the importance of the background ecosystem, and this will depend partly on the migration of tree species (which may be very slow to migrate [Svenning *et al.*, 2004]). Nothing seems to be known about nematode range migration potential in general. It is at least plausible that nematode range adjustment to climate might take place over a few decades on a mountain system, where climate zones would shift only a few hundred meters in response to a several degrees rise in temperature. Similar migrations in treelines have been observed in various locations over past 100-150 years (Serreze *et al.*, 2000). Such uncertainty makes clear that further observational, experimental and modelling work is needed.

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