Developing a qPCR-based Molecular Technique for Nematode Community Analysis

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII AT MANOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
TROPICAL PLANT PATHOLOGY

May 2012
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Acknowledgement

I thank the members of my committee, Drs. Brent. S. Sipes, K.-H. Wang, Robert M. Paull, Ming-Li Wang, Roxana Cabos and Ruey-Shang Chen for their advice, comments, assistance and ideas during the course of my study. I especially thank Dr. K.-H. Wang for her assistance in identifying free-living nematodes, and advise on conducting nematode community analysis, Dr. Ruey-Shang Chen for research ideas and Dr. Brent S. Sipes for his support and suggestion on my overall dissertation progress and my graduate studies.

I would also like to thank Donna Meyer, Michael Young for their technical assistance, and the rest of the University of Hawaii Nematology Lab members for all their support.

Finally, I also like to thank my family and friends for their un-conditioning support and accompanying me throughout my graduate studies. I made it!
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Abstract

Nematodes are good indicators for soil health. However performing nematode community analysis is laborious and technically challenging. This research seeks to develop a qPCR-based molecular tool for nematode community analysis. qPCR detecting 18S rDNA offered one approach to identify and quantify free-living nematodes. Owing to too many unpredictable nematode genera across soil ecosystems, one strategy is to develop universal qPCR markers selective for key nematode guilds (Ba1, Ba2, F2, P4, Om4, Om5, and P5) that are most critical for nematode faunal analysis. Universal qPCR primers were successfully being identified for all of these guilds except for Ba1. Two primers were needed for F2; Om4, Om5, and P5 cannot be differentiated and were thus being combined as Om4/Om5/P5 by one primer. These primers were then verified by BLAST and then run through artificial nematode mixture sample composed with known nematode guilds. The results confirmed the validity of these universal primers. The next logical step was to run these qPCR to nematodes collected from four natural ecosystems: forest, organic, pineapple field, and beach sites. Visual nematode identification on these four systems was being conducted to compare results. Two qPCR standard curves (plasmid DNA and genomic DNA) were used to obtain nematode abundance of the four ecosystems. Since both DNA standard curves did not estimate nematode abundance comparable to the visual count, ranking of nematode community indices of the four ecosystems were compared between molecular and the visual methods. While the ranking calculated by the plasmid DNA standard curve of qPCR assay were not consistent with most of the nematode community indices calculated by visual method, 4 out of 8 nematode indices estimated by the gDNA standard curve were relatively consistent. This
research provided universal nematode guild qPCR primer sets and initial protocol of qPCR-based molecular tool for soil nematode community analysis. Further research need to be conducted on better estimation of nematode abundance, richness and diversity. More universal primers selective for Ba1, Ba3, F3, P3, also Om4, Om5, and P5 individual primers are needed.
Chapter 1

Introduction

Soil Health and Soil Ecosystem Management

Soil is a complex ecosystem which supports a heterogeneous, discontinuous and structured environment dominated by a solid phase. The morphological, physical, chemical, and mineralogical characteristics of the solid phase influence the living components of the ecosystem (Stotzky, 1990; Birkeland, 1999). As a living ecosystem, soil sustains a variety of biota outside the matrix while also maintaining and enhancing the quality of life, water and air. Plant and animal productivity can be greater in healthy soils compared to unhealthy soils (Doran, 2000; Eldor, 2006).

The outbreak of soil-borne disease can be considered an indicator of instability and poor ecosystem health. Therefore, a link between soil health, the ability of the biological community to suppress plant pathogens, the population density of plant pathogens in soil, and ultimately disease incidence and severity is likely to exist (van Bruggen, 1996). An unhealthy soil will potentially result in an outbreak of soil-borne diseases. Wang and
McSorley (2005) proposed a model of soil ecosystem management where soil pathogen and beneficial soil organisms should be managed concurrently (Wang and McSorley, 2005). The concept of soil ecosystem management is based upon understanding the biology of soil pests and pathogens. One should select agricultural practices that suppress pests while enhancing beneficial soil organisms that could enhance plant health (Wang, personal communication). To suppress pathogens, soil ecosystem management is essential. The stability and resilience of the ecosystem (which henceforth will be referred to as soil health) depends on maintaining a diverse soil food web (Gregorich, 1997).

**Soil Food Web**

Soil food webs are composed of a myriad of organisms, ranging from single-celled bacteria, algae and protozoa to multi-celled mites, earthworms, collembola and nematodes. The soil food web transverses prokaryotes to eukaryotes, yet provides reservoirs of minerals and nutrients. The soil food web decomposes organic matter, detoxifies pollutants, modifies soil structure, and regulates the abundance of pests and other opportunistic species. (Griffiths, 1994; Kennedy, 1995; van Stracilen, 2009). The diversity of soil organisms usually represents the health of a soil ecosystem. In terms of biodiversity, soil should have a variety
of taxonomic groups, including algae, bacteria, diatoms, fungi, protozoa, nematodes, earthworms and arthropods to be considered healthy (Neher, 2001).

Characteristics of a healthy soil are biological diversity, ability to maintain soil nutrient cycling, stability in response to disturbance or stress, ability to suppress multiple pests and pathogens, and the improvement of plant health (Wang, 2005). Many different approaches are available to assess and characterize soil health. Examining biodiversity is one common approach. A conference on ‘Soil Health: Managing the Biological Component of Soil Quality’ highlighted the potential of various soil organisms as soil health indicators (Doran, 2000). Taxonomic groups form a complex food web of many trophic levels. However, significant semantic, conceptual and technical problems limit the usefulness of biodiversity indices as indicators of soil health (Neher 2001). For instance, some indices reveal the total taxon proportions without differential weight for qualitative difference among taxa, which render the indices insensitive to taxon composition (Jongman, 1995). In addition, soils with 100% exotic or 100% native species could have identical diversity values, thus biodiversity index alone does not describe soil health conditions sufficiently (Neher 2001).
Since the 1990s, it has been proposed that soil microorganisms can be used as indicators of soil health (Smith, 1990; Karlen, 1997; Doran, 2000). When using microorganisms as bioindicators, besides abundance and biomass, the bioactivity and community structure of the organisms are also very important measurements. Measurement of disrupted soil processes, decreased bacterial or fungal activity, decreased fungal or bacterial biomass, changes in the ratio of fungal to bacterial biomass, decreases in the number or diversity of protozoa, as well as a change in nematode numbers, nematode community structure or nematode maturity index can indicate a problem in the soil ecosystem long before soils become unproductive (Bongers, 1990; Klopatek, 1993)

**Nematode as Soil Health Indicator**

Among soil microorganisms, nematodes transcend and occupy multiple levels in the soil food web. Nematodes are ubiquitous and play a key role in mediating soil nutrients by feeding on fungi, bacteria, cyanobacteria, green algae, plant roots, and other soil fauna with diverse feeding habits (Yeates, 1993; Bardgett, 1999). Besides, nematodes have life strategies ranging from colonizers to persisters (Neher, 1996; Bongers, 1998; Neher, 1998; Bongers, 1999). Among taxonomic groups, nematodes contribute to nitrogen mineralization indirectly
by grazing on decomposer microbes, excreting ammonium and immobilizing nitrogen in live biomass (Ingham, 1985). Nematodes are sensitive to environmental changes. Furthermore, nematodes represent an important part of the soil microfauna that directly and indirectly affect the size, activity and diversity of the other soil microflora (Neher, 2001; Waite, 2003). Nematode taxa are well classified, and their morphology reflect their feeding behavior, allowing functional classification (Bongers, 1998; Neher, 2001). In other words, nematodes are excellent indicators of the structure and function of the soil ecosystem (Neher, 1996, 1998). Similarly, detailed literature exists for the use of nematodes as indicators in freshwater environments and marine ecosystems (Hodda, 1986; Lambshead, 1986; Bollag, 1990; Warwick, 1990; Neilson, 1996; Beier, 2001, 2003).

In order to evaluate sustainability of agricultural practices, assessment of soil health is required (Karlen, 1997; Doran, 2000). An assessment that utilizes soil nematode community analysis provides the depth and breadth needed to ascertain soil health. Nematodes can be utilized as soil health indicators because of their abundance and ubiquitousness. Also nematodes have diverse feeding behaviors and life strategies, as well as interactions with other soil organisms. Therefore nematodes can be used to assess the abiotic and biotic components of soil and further measure function and structure of the soil ecosystem.
Nematode Functional Guilds

Nematode C-P value

Functional guilds of nematodes have been defined by nematode feeding types (bacteria-feeding, fungal-feeding, omnivore, predatory and plant-feeding) (Yeates, 1993). Furthermore, nematode functional guilds have been refined into colonizer-persister (c-p) class which reflects life-history strategies in c-p value, making this group of organisms particularly useful for soil food web analysis (Bongers, 1990). Colonizers and persisters are extremes on a scale from 1 to 5 respectively. Colonizers are nematodes that increase in number rapidly under favourable conditions, have short life-cycles and high colonization ability. Colonizers have tolerance to disturbance, eutrophication, and anoxysbiosis. This is comparable to r-strategies (in the loose sense), usually they are dominant within samples (Bongers, 1990). Additionally, functional guilds of nematodes are indicators of other organisms with similar ecological function and thus of ecosystem responses (Bongers, 1990; Wardle, 1993; Todd, 1996; Bongers, 1998, 1999, 1999; Yeates, 1999). The nematode taxons have been re-organized by combining the feeding habits of soil nematodes from Yeates with the c-p value (Yeates, 1993; Bongers, 1995, 1998, 1999, 1999).
For instance, the nematode guild \textit{cp-1} is typified by short generation time, production of many small eggs resulting in explosive growth and a high reproductive rate under conditions where microbial activity is high. Guild \textit{cp-1} members are relatively tolerant to pollution-induced stress. These enrichment opportunists show a phoretic relation with insects and other vectors and are only active under transient conditions of high microbial activity. Guild \textit{cp-1} members form dauer larvae as microbial activity decreases (Bongers, 1998). This group is composed of rhabditid, diplogastrid, and panagrolaimid bacterial feeders (Bongers, 1998).

Nematode guild \textit{cp-2}, similar to the \textit{cp-1} group, has a short generation time and a high reproduction rate, \textit{cp-2} members do not form dauer larvae. They occur under food-rich as well as food-poor conditions and are very tolerant to pollutants and other disturbances. This group is composed of the smaller tylenchids, mainly feeding on epidermal cells. The fungal feeding aphelenchoids, anguinids and the bacterial feeding cephalobids, plectids and monhysterids comprise the \textit{cp-2} guild (Bongers, 1998). \textit{Cp-3} guild nematodes have characteristics between \textit{cp} guild 2 and 4. \textit{Cp-3} members have a longer generation time and are relatively sensitive to disturbances. \textit{Cp-3} is composed of the bacterial feeding teratocephalids, the Araeolaimida and Chromadorida. The larger tylenchid nematodes that
feed on deeper cell layers in the roots are assigned to the \( cp-3 \) guild. The diphtherophorids, assumed to feed on fungi, and the carnivorous tripylids are also categorized to the \( cp-3 \) guild.

Persisters are comparable to \( k \)-strategies (sensuato). These nematodes have a low reproduction rate, long life-cycle, low colonization ability and are more sensitive to disturbances. In general, persisters live in habitats that are stable for long durations. The \( cp-4 \) includes small dorylaimids and large non-dorylaimids. These nematodes are characterized by a long generation time, permeable cuticle and sensitivity to pollutants. This group is composed of larger carnivores, the bacterial feeding Alaimidae and Bathyodontidae, the smaller dorylaimid nematodes and the plant feeding trichodorids (Bongers, 1998). Nematode guild \( cp-5 \) is composed of large dorylaimid nematodes: omnivores, predators and plant feeders with a long life span and low reproduction rate; both probably a corollary to low metabolic activity. \( Cp-5 \) guild members produce few but large eggs and their motility is low. With a permeable cuticle the nematodes are very sensitive to pollutants and other disturbances (Bongers, 1998).
Nematode Feeding Groups

Bacterivorous nematodes feed on bacteria and are usually present in high abundance in the soil. The stoma of this nematode is a hollow tube for ingestion (Yeates, 1993). Common genera of bacteria feeding nematodes are *Rhabditis* and *Caenorhabditis*. These nematodes are beneficial in the decomposition of organic matter. Bacterial-feeding nematodes contribute to nitrogen mineralization by grazing on and dispersing bacteria (Anderson, 1981; Freckman, 1988; Bouwman, 1994; Griffiths, 1994). Increase in the abundance of bacterivorous nematodes often indicates an increase in readily decomposing organic matter of plant or animal origin (Wasilewska, 1974; Sohlenius, 1984; Wasilewska, 1985; Dmowska, 1988; Freckman, 1988; Freckman, 1993; Griffiths, 1994; Todd, 1996; Wasilewska, 1999). The majority of bacterivorous nematodes are within the *cp*-1 guild Ba1 with Diplogasteridae, Tylopharyngidae, Rhabditidae, Panagropaimidae and Bunonematidae forming the group; The Ba2 group (*cp*-2 guild members) includes Monhysteridae, Xyalidae, Plectidae, Leptolamidae and Cephalobidae.

Fungivorous nematodes feed on fungi. These nematodes employ a stomatostyle or odontostyle to puncture and feed on fungal hyphae. Many members of the order *Aphelenchida*
fall into this group, especially species of *Aphelenchus* and *Aphelenchoides*. Similar to bacterivores, fungivorous nematodes are very important in decomposition. In soil nutrient cycling, fungivorous nematodes function by mineralizing soil nutrients like bacterivorous nematodes. Moreover, fungivorous nematodes can indicate the pH level of soil (Porazinska, 1999). Compared to other free-living nematodes, increasing abundance of fungivorous nematodes is indicative of soil nutrient enrichment. The majority of fungivorous nematodes fall into the nematode guild F2. Families within F2 include the Anguinidae, Neotylenchidae, Aphelenchidae and Aphelenchiodidae.

Herbivorous nematodes are those that feed on plant. Plant-parasitic nematodes are in this group, which includes many members of the order Tylenchida, as well as a few genera in the Aphelenchida and Dorylaimida. Different species of herbivorous nematodes can inhabit and infect most living parts of plants, including flower buds, stems and roots (Yeates, 1993). Plant-parasitic nematodes are characterized by a needlelike stylet (tylenchoid stomatostyle or dorylaimoid odontostyle). The majority of herbivorous nematodes fall into the nematode guild H2 and H3. Families within H2 include Tylenchidae and Paratylenchidae. The H3 group includes Hemicycliphoridae, Hoplolaimidae, and Meloidogynidae.
Omnivorous nematodes usually feed on more than one type of food material. Some omnivorous nematodes may ingest fungal spores and bacteria cells, while occasionally feeding on other nematodes smaller than themselves. For instance, some members in the order Dorylaimida, which includes *Aporcelaimellus* spp., *Eudorylaimus* spp. and *Mesodorylaimus* spp., may feed on fungi, algae and other animals. In the omnivores, Om4 and Om5 groups compose the majority of nematodes, Om4 contains the Qudsisanematidae, Nordiidae and Dorylaimidae whereas Aporoelaimidae is assigned to Om5.

Predatory nematodes feed on many kinds of soil microorganisms including plant-parasitic nematodes. Predatory nematodes also play a role in soil nutrient cycling because they can mineralize nutrients fixed in their prey (Khan, 2007). Predatory nematodes act as conduits by which resources pass from lower to higher trophic levels (Wardle, 1993). The majority of predatory nematodes belong to four taxonomic groups of nematodes, Mononchida, Dorylaimida, Diplogasterida and Aphelenchida. Each group has its own type of feeding apparatus, feeding mechanisms and food preferences (Khan, 2007). The Mononchids possess an open, sclerotized buccal cavity, which is often armed with a tooth, puncturing teeth, numerous small grasping teeth or a combination of the above. Dorylaimid predators have odontostylets which are used to puncture their prey. As the most ubiquitous group of
predatory nematodes, Aphelenchid predators possess a fine needle-like aphelenchid stylet that can pierce the cuticle of their prey and inject digestive enzymes into the prey body, paralyzing the prey almost instantly (Hechler, 1963; Wood, 1974). The majority of predatory nematodes are found in $cp$ guilds P4 and P5 guild. P4 guild includes Mononchidae, Anatonchidae, Choanolamidae and Ironidae; whereas the P5 guild includes Nygolaimidae and Actionlaimidae.

The significance of different nematode guilds used in the nematode community indices calculated is used to indicate soil health. In this proposed dissertation, the focus will be on investigating nematode community indices using a molecular approach.

**Nematode Identification for Community Analysis**

**Nematode Community Analysis**

Several nematode community indices have been developed to indirectly analyze soil conditions, including richness, dominance, diversity, maturity index (MI), enrichment index (EI), structure index (SI) and channel index (CI). The $c-p$ scale is used to calculate maturity index (MI). MI is the weighed (mean) $c-p$ value for all the nematodes in a soil community. A
maturity index for free-living taxa (MI) may be viewed as a measure of disturbance, with smaller values being indicative of a more disturbed environment and larger values characteristic of a less disturbed environment. The MI decreases with increasing microbial activity and pollution induced stress which usually occurs when organic matter is freshly incorporated into the soil. The increased microbial activity provides a favorable environment for low c-p value nematodes which feed on microorganisms therefore MI indicates the majority of c-p values for the nematodes in the soil community (Bongers, 1999, 1999).

Nematode functional guild has also been used to develop more indices. Relative weighted abundance of nematode guilds was utilized to describe soil food web conditions-enriched, basal, and structured (Ferris, 2001). The Basal component (b) is the fungal and bacterial feeders in the cp-2 guild and is calculated as \( b = kbnb \) where \( kb \) is the weighted constant for the guild and \( n \) is the number of nematodes in Ba2 and F2. Enrichment \((e)\) component is calculated using nematode guilds indicative of enrichment (bacterivores of \( cp-1 \), and fungivores of \( cp \ 2 \)). The structure component \((s)\) is calculated using guilds of bacterivores of \( cp-3-5 \), fungivores of \( cp \ 3-5 \), omnivores of \( cp-3-5 \), and predatory nematodes of \( cp-2-5 \). Enrichment index is then calculated as \( 100 \times \frac{e}{e + b} \), and SI is calculated as \( 100 \times \frac{s}{s + b} \). CI, the Channel Index is calculated from Ba1 and F2 guilds with their respective
weightings as $100 \times (0.8 \ F_2/(3.2 \ Ba_1 + 0.8 \ F_2)$ (Ferris, 2001). Overall, a higher EI indicates a nutrient enriched environment. Higher SI indicates a structured or undisturbed community and a higher CI indicates a community with higher proportion of fungal decomposition than bacterial decomposition, i.e., a more stressful condition. Moreover, fungivore to bacterivore (F/B) ratios were calculated to characterize decomposition and mineralization pathways, using the F/B ratio of Freckman and Ettema and the F/(F+B) ratio of Neher (Freckman, 1993; Neher, 1999). This ratio describes the decomposition pathway in detritus food webs. Smaller ratios are associated with faster rates of decomposition and nutrient turnover. Thus, calculation of nematode community indices provides information on soil health status.

**Traditional and Molecular Nematode Identification Methods**

Traditional nematode identification relies mainly on morphology as observed with microscopes (Roman, 1969; Frederick, 1978). However, biochemical methods such as electrophoresis, chromatography, monoclonal antibodies and molecular methods are available now. Molecular methods used to identify nematodes include PCR-based methods (Porazinska, 1999; Gasser, 2001; Hernandez, 2003; Waite, 2003; Powers, 2004; Blok, 2005;
Griffiths, 2006; Holeva, 2006; Ward, 2006; Watzinger, 2006; Porazinska, 2009), genome structure (Floyd, 2002; Gorokhova, 2002; Shufran, 2003) and Denaturing Gradient Gel Electrophoresis (DGGE) assays (Foucher, 2002; Powers, 2004). Molecular techniques have been used in ecological characterization by using nematode life cycle, feeding habits, host plant reactions, and profiles of reproductive traits (Eisenback, 1988; Riggs, 1988; Yeates, 1993). Molecular techniques have also been used in nematode evolution programs, genetic phenotypes, region of nematodes and co-evolution between host plants and nematodes (Henning, 1966; Moss, 1970; Siddiqi, 1983).

**Disadvantage of Traditional Identification Methods**

Nematodes are easily placed into appropriate nematode guilds by expert observers. However, this experience can be difficult to find or develop. Identification of individual nematodes under the microscope followed by calculation of nematode abundance, diversity and various other nematode community indices are tedious procedures used in traditional nematode community analysis. Most of these indices require the knowledge of the family or even genus levels of each nematode identified for their calculation (Bongers, 1998; Ferris, 2001). The reliance on visual morphological features observed by experts and the time
required are limiting factors to the greater adoption of nematodes as soil health indicators.

Nematodes can be morphologically similar but genetically divergent. Moreover, for accurate identification of certain nematodes, adult specimens are required. However, nematode populations often are comprised of a large percentage of juveniles.

**Advantages of Molecular Identification Methods**

There are advantages using molecular methods for nematode identification and diagnosis. Identification methods for nematodes must provide accuracy, speed, reliability, affordability, and if possible, enable characterization of specimens new to science. Molecular-based identification may overcome the limitations on community analysis adoption imposed by the time associated with visual nematode identification and the expertise needed (Table 1.1). Besides, molecular identification methods can apply to all life stages. For example, molecular methods have proven to be reliable in identifying infective juveniles of *Meloidogyne mayaguensis* in the soil (Brito, 2004). Most nematodes are approximately 1 mm in size and comprised of 1000 to 2000 cells (Powers, 2004). Conveniently, this size is suitable for a PCR reaction with minimum effort required for DNA extraction. Also, there are no recalcitrant structural components that must be separated or circumvented to extract nematode DNA.
Molecular approaches are becoming more widely applied to fulfill new demands, in part because of the relative simplicity of their application in the laboratory (Powers, 2004). This is especially the case as currently training in classical taxonomic techniques is in decline while that in molecular methods is increasing.

Table 1.1. Comparison of estimated time required to identify nematodes for 50 samples using traditional and PCR-based methods.

<table>
<thead>
<tr>
<th>Step</th>
<th>Traditional Tool Estimate Time (100 samples)</th>
<th>Molecular Tool Estimate Time (100 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection</td>
<td>Same</td>
<td>Same</td>
</tr>
<tr>
<td>Extraction</td>
<td>4 Hour</td>
<td>8 Hour</td>
</tr>
<tr>
<td>Identification</td>
<td>100 Hour</td>
<td>10 Hour</td>
</tr>
<tr>
<td>Total</td>
<td>104 Hour</td>
<td>18 Hour</td>
</tr>
</tbody>
</table>
Challenge and Future Prospect of Molecular Methods for Nematode Community Analysis

The identification of representative specimens of organisms is critical. Ironically, this crucial first step is often the least critically examined step in diagnostic studies (Stevens, 2003). The construction of the rapidly growing molecular database must include a methodology to evaluate the quality of the data. Without adequate documentation or designation of the voucher material, there is no significance to a DNA sequence. Ideally, each DNA sequence in GenBank or each published PCR-RFLP gel pattern should be accompanied by information on morphometric measurements, collection site, and even a picture of the voucher material. The voucher material may consist of DNA, digitized images, videos or a portion of the original specimen (De Ley, 2002). This has been the standard for traditional, morphological-based diagnostic research, and there should be no relaxing of these standards for molecular studies.

Selecting a reliable DNA region to work on is also important. Amplification and sequencing of the diagnostic regions (i.e., rapidly evolving regions of SSU (small subunit) ribosomal DNA and LSU (large subunit) ribosomal DNA) of single nematode specimens
have resulted in the development of extensive public DNA-sequence databases that are available for BLAST-match searching (Powers, 2004). Although DNA-based databases are strongly biased towards plant-parasitic nematodes, the utility of these databases for free-living nematode identification has been developed in more recent years (De Ley, 2005).

Different molecular methods have been used to identify nematodes. For example, PCR-based methods, including randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) can produce sequence characterized amplified regions (SCARs) and can be used to characterize different nematodes. Foucher and Wilson (2002) used denaturing gradient gel electrophoresis (DGGE) to distinguish nematode species present in a mixed culture to analyze nematode communities by separating PCR products based on sequence differences. After sequencing, the sequences similarity was analyzed and saved as a record for nematode richness (Foucher, 2002; Waite, 2003). PCR-DGGE also has been used to obtain relative-quantification of nematode community by using EtBr or SYBR Green dye staining the DGGE gel (Okada, 2008). Moreover, terminal restriction fragment polymorphism (T-RFLP) is proposed as a means of nematode community analysis and can be used to profile nematode communities under different agricultural regimes (Griffiths, 2006, 2012). Furthermore, real-time quantitative PCR (qPCR) and multiplex real-time PCR
(qPCR) extend the PCR based method to the quantification of nematodes (Madania, 2005; Holeva, 2006; Nakhla, 2010). Sequencing of nematode rDNA has also been used to develop a database for nematodes.

However, when applying molecular methods to nematode community analysis, it is important that molecular techniques need to reflect the relative abundances of the particular species or functional guilds (Yeates, 1999). There is more and more research that has compared molecular and morphological techniques in nematode community analysis. Griffiths et al. (2006) combined morphological and molecular sequencing to establish the potential for analyzing nematode communities by molecular biological characterization, but found that nematodes from the Rhabditida and Tylenchida were underrepresented in the molecular characterization when compared to the morphological method (Griffiths, 2006). Also, Okada and Oba (2008) compared the results of nematode communities between DGGE and morphological identification (Okada, 2008). Their study on the effect of copper contamination on soil nematode diversity used PCR-DGGE and morphological analysis. They found that PCR-DGGE could give more information on nematode genera, and the intensity of the bands could reflect the relative abundance of nematode genera in the assemblage (Wang, 2008). Furthermore, the T-RFLP (terminal restriction fragment length
polymorphism) has been conducted for nematode community analysis. A fluorescently labeled PCR primer was used in PCR assay, and run in the capillary sequencer after the restriction enzyme digestion. This method is able to give the information of nematode richness, relative abundance (Griffiths, 2006). Moreover, when compared the results obtained using DNA sequence-based molecular approach to results obtained using a traditional, microscopy-based approach, it was found that the results were broadly similar (Hamilton, 2009). Yet, the constraint of molecular methods in nematode community analysis is most molecular methods cannot provide quantification of nematode communities but mostly richness, diversity of them.

PCR is extensively applied to the identification of nematodes because the nematode may simply be crushed in a droplet of water and a portion of the solution added directly to a PCR reaction (Powers, 1993). Multiple amplifications (5–10) can routinely be achieved from a single nematode. The frozen solution from a crushed nematode can be stored indefinitely and thereby serve as a DNA voucher for future analysis. Among different kinds of PCR methods, real-time quantitative PCR (qPCR) is the most suitable PCR method when conducting nematode DNA quantification for nematode community analysis. qPCR allows analysis of the quantities of DNA in multiple samples in the same time. Even a limited amount of DNA
material is sufficient to obtain highly specific and reproducible results in qPCR, qPCR is widely applied in microbiology for detection and quantification of viruses (Watzinger, 2006), bacteria (Ward, 2006) and other microorganisms as well as genetically modified organism components in transformed organisms (Hernandez, 2003). Recently, detection of the nematodes *Globodera pallida* and *Heterodera schachtii* using qPCR assay with SybrGreen dye was conducted. Detection of *G. rostochiensis* and *G. tabacum solanacearum* using TaqMan dye have been described (Madania, 2005; Nakhla, 2010). Therefore, a qPCR method will be utilized to quantify the DNA of the different nematode guilds Ba1, Ba2, F2, P4, P5, Om4, and Om5 in the soil sample. The results should be revealing the abundance of nematode guilds, and be applicable to the nematode community analysis.

**Nematode Genome Region as Molecular Method Targets**

Nematode identification by PCR-based methods include (a) ribosomal DNA (internal transcribed spacer (ITS)1, ITS2, 18S, D2-D3, 5S, and intergenic spacer (IGS) region), (b) 5S-spliced leader, (c) major sperm protein, (d) mtDNA (cytochrome oxidase (CO)IIIRNA and COI), and (e) satellite DNA (Blok, 2005). Different purposes can be achieved with PCR by targeting different parts of the nematode genome. Ribosomal DNA has provided the basis
of most of the published molecular identification and diagnosis of nematodes (Crease, 1991; Hillis, 1991; Guschin, 1997; Gorokhova, 2002; Shufran, 2003). The large intergenic spacer (IGS) which separates the 28S and 18S coding regions and the repetitive regions inside have been used to identify nematodes between or within species (Powers, 1997; Gasser, 2001; Powers, 2004). The highly conserved regions, 18S, 5.8S and 28S rDNA are suitable for designing universal primers whereas sufficient sequence divergence and stability in ITS region allows species and subspecies identification (White, 1990; Hillis, 1991; Hyman, 1991; Floyd, 2002). Eyualen and Blaxter in 2003 characterized five morphologically identical populations of Panagrolaimus into two species by sequencing 18s rDNA. Furthermore, the ‘molecular operational taxonomic unit (MOTU)’ approach, which was developed for sequencing PCR amplicons from individual nematodes, is also applicable to community analysis (Floyd, 2002; Eyualem, 2003). In this dissertation, qPCR will be utilized to quantify different nematode indicators’ 18S rDNA. Universal primer sets can be developed from representative nematode taxon of different nematode guilds, which will quantify the abundance of representative nematode taxon and further indicate the nematode guilds’ abundance within the soil sample and enable the calculation of the nematode community indices.
qPCR assay and Nematode Community Analysis

The main constraint of using molecular tools for nematode community analysis is the lack of efficient tools to quantify the abundance of nematodes. Among the nematode community indices such as percentages of trophic groups, MI (Bongers, 1998), SI, EI, and CI (Ferris, 2001), quantifying the abundance or relative abundance of nematodes in each trophic group or nematode guild is essential. Although molecular tools allow identification of nematodes to a more defined level and at a faster pace than the traditional morphological identification method, the main challenge is to quantify the abundance of nematodes in each nematode guild molecularly. qPCR has been applied to many nematode identification and quantification studies (Madania, 2005; Holeva, 2006; Ward, 2006; Watzinger, 2006; Nakhla, 2010) but has not been utilized for nematode community analysis with nematode guilds or nematode trophic groups. While the study of soil nematode diversity by PCR-DGGE has found that the intensity of the bands could reflect the abundance of nematode genera in the assemblage (Wang, 2008), qPCR offers another approach to measure the abundance of nematode. However, performing qPCR for each nematode taxon that might be present in one nematode community is tremendously costly and uncertain of completion. This is because an unlimited number of PCR primer sets are required to ensure completion of this analysis.
Hypothesis and Objectives

The overall goal of this dissertation is to utilize a qPCR assay to study nematode communities by quantifying representative nematode taxon from different nematode guilds. No qPCR approach has been targeted to quantify nematode community indices. This dissertation developed a qPCR protocol to allow quantification of nematode community indices.

Performing qPCR for each nematode taxon in a nematode community is tremendously costly and carries uncertain amount of selection of primers. Selecting representative nematode taxon crucial to distinguish soil health conditions might offer one solution. One taxon per nematode guild is the goal. The essential nematode guilds for nematode community indices include Ba1, Ba2, F2, Om4, Om5, P4, and P5. The results from the molecular nematode community analysis based on qPCR will be compared to those from the visual identification method. Specific objectives of the research were:
Objective 1: Develop universal primer sets for nematode indicators within the nematode guilds- Ba1, Ba2, F2, P4, P5, Om4, and Om5.

Objective 2: Determine ability of universal qPCR primer sets to indicate for nematode guild Ba1, Ba2, F2, P4, P5, Om4, and Om5.

Objective 3: Evaluate and compare real-time quantitative PCR (qPCR) and visual nematode identification for nematode soil community analysis.
Literature Cited


Frederick, J.J. and Tarjan, A.C., 1978. Variability in measurements made of same nematode specimen by various observers or by one observer on different days. *Nematologica* 24, 476-479.


Chapter 2

Develop universal primer sets for nematode indicators within the nematode guilds- Ba1, Ba2, F2, P4, P5, Om4, and Om5

Among all the molecular tools applied to nematode identification, PCR has come to be extensively used because of its speed and convenience (Powers, 1993). Among different types of PCR methods, real-time quantitative PCR (qPCR) is the most suitable technique for quantification of nematode DNA for nematode community analysis (Madania, 2005; Nakhla, 2010). qPCR allows analysis of the quantity of DNA in multiple samples at the same time (Powers, 2004; Blok, 2005; Madania, 2005; Nakhla, 2010). qPCR is widely used for detection and quantification of viruses (Watzinger, 2006), bacteria (Ward, 2006) and other microorganisms (Hernandez, 2003). However, the application of qPCR is still restricted to specific nematode identification or determination of particular nematode abundance. Madani et al. (2005) emphasized that precise identification and knowledge about the number of nematodes in field soil are necessary to develop effective integrated pest control. Madani also reported on qPCR primers for *Globodera pallida* and *Heterodera shachtii*. Recently, qPCR primers were reported for *Meloidogyne chitwoodi* and *Meloidogyne fallax* (Zijlstra, 2006)
and the root-lesion nematode *Pratylenchus penetrans* (Sato, 2007). Detection of *G. rostochiensis* and *G. tabacum solanacearum* has been achieved using TaqMan dye (Madania, 2005; Toyota, 2008; Nakhla, 2010). The qPCR assay could be used for nematode community analysis but is challenged by the requirement of individual primer sets for each taxa. Through the understanding of the key nematode community indices developed and revised by several nematode ecologists (Neher, 2001; Ferris, et al., 2001), the current research project proposed to develop nematode community analysis using qPCR targeting on key nematode guilds.

The 18S rDNA was targeted as the best candidate DNA sequence area since the region is highly conserved. The 18S, 5.8S and 28S rDNA are suitable for designing universal primers because they showed much greater conservation than the transcribed and nontranscribed intergenic regions (Hillis, 1991; Gorokhova, 2002; Blok, 2005). Furthermore, the 18S rDNA nematode information in the database is richer than the 5.8S and 28S data sets (White, 1990; Hillis, 1991; Hyman, 1991).

Although this selective approach might not allow a comprehensive detection and quantification of all nematode taxa present in a soil community, the overall goal is to develop molecular tools to compare soil health conditions across ecosystems. Thus, the objective of this research was to identify universal qPCR primers nematode guilds Ba1, Ba2, F2, P4, P5,
Om4, and Om5, respectively.

**Materials and Methods**

$q$PCR Primer Design

To find a suitable multicopy region for primer design, the public database GenBank in NCBI (National Center of Biotechnology Information) was screened. Representative nematodes were selected from nematode guilds Ba1, Ba2, F2, Om4, Om5, P4 and P5 based on common occurrence in various soil ecosystems in Hawaii (Wang et al., 2009, 2011; Marahatta 2010). The selected representative nematode family for Ba1 was Rhabditidae, Ba2 the Cephalobidae, F2 the Aphelenchidae and Aphelenchoididae, Om4 the Dorylaimidae, Om5 the Aporocelaimidae, P4 the Mononchidae, and P5 the Nygolaimidae (Table 2.1).
Table 2.1 Representative taxon and number of sequence sets collected from GenBank of NCBI of nematode guild Ba1, Ba2, F2, Om4, Om5, P4 and P5

<table>
<thead>
<tr>
<th>Functional Guild</th>
<th>Representative Taxon</th>
<th>Sequence Sets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba1</td>
<td>Rhabditidae</td>
<td>185 sequence sets</td>
</tr>
<tr>
<td>Ba2</td>
<td>Cephalobidae</td>
<td>188 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Eucephalobus</em>, <em>Acrobelodies</em> and <em>Acrobeles</em></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>Aphelenchidae</td>
<td>74 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Aphelenchus</em> and <em>Filenchus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aphelenchoididae</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aphelenchodies</em></td>
<td></td>
</tr>
<tr>
<td>Om4</td>
<td>Dorylaimidae</td>
<td>56 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Mesdorylaimus</em>, <em>Dorylaimus</em>, <em>Eudorylaimus</em> and <em>Aphelenchus Eucumenicus</em></td>
<td></td>
</tr>
<tr>
<td>Om5</td>
<td>Aporcelaimidae</td>
<td>29 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Aporceloimellus</em> and <em>Paraxonchium</em></td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>Mononchidae</td>
<td>18 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Prionchulus</em> and <em>Mononchus</em></td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>Nygolaimidae</td>
<td>10 sequence sets</td>
</tr>
<tr>
<td></td>
<td><em>Paravulvus</em> and <em>Nygolaimus</em></td>
<td></td>
</tr>
</tbody>
</table>
**Ba1:** Sets of Rhabditidae 18S rDNA sequences (Table 2.1) were collected from the database and aligned using Clustal W ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)). A conserved region was defined and aligned with Ba2 guild sequences to determine a Ba1 specifically conserved region (~200 bp). However, the determined Ba1 conserved region could also detect Ba2 nematodes. A different strategy of random primer cloning using hexamer primers (Feinberg, 1993) that randomly anneal to template DNA or RNA with polymerase or reverse transcriptase in reaction was employed to develop universal primer sets for Ba1 nematode guild.

**DNA Extraction**

DNA from Ba1 nematodes were extracted using the PureLink™ DNA Extraction Kit (Invitrogen) with a protocol modified with bead-beating and heating steps (Donn, 2008). Ba1 nematodes (50) were collected from soil samples, visually identified, and picked into a sterilized 1.5 ml eppendorf tube filled with 200μl distilled water. The tube was stored until needed. Samples were thawed, placed on ice and bead-beated with 100 μg 1mm glass beads under room temperature (Sigma, USA) for 2 minutes, after beating, 180 μl of Genomic Digestion Buffer (PureLink™ DNA Extraction Kit, Invitrogen, USA) with 20 μl of Proteinase K added to the tube. The tube was then heated to 95°C for 3 minutes. A lysate was prepared with PureLink™ Genomic Lysis/Binding Buffer (200 μl) and an equal volume of
ethanol which was added to a PureLink™ Spin Column. The column was centrifuged at 10,000 × g for 1 minute at 25°C. The collection tube and the spin column were placed into a clean PureLink™ collection tube. A 500 μl wash buffer I was prepared with ethanol and added to the column. The column was centrifuged at 25°C at 10,000 × g for 1 minute and the collection tube discarded. The spin column was placed into a clean PureLink™ collection tube. A 500 μl wash buffer II was prepared with ethanol and added to the column. The column was centrifuged at 14,000 × g for 3 minutes at 25°C and the collection tube discarded. The spin column was placed in a sterile 1.5-ml microcentrifuge tube. 40 μl of PureLink™ Genomic Elution Buffer was added to the column which was then incubated at 25°C for 1 minute. The column was centrifuged at 14,000 × g for 1 minute at 25°C. To recover additional nematode DNA, a second elution using the first elution with DNA was performed. The column was centrifuged at 14,000 × g for 1.5 minutes at 25°C. The column was removed and discarded. The purified nematode gDNA was stored at -20°C.
Table 2.2 Universal random primer numbers in this study (Protech Technology Enterprise Co, Taiwan)

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>211</td>
<td>5' GAAGCGCGAT 3’</td>
</tr>
<tr>
<td>212</td>
<td>5' GCTGCGTGAC 3’</td>
</tr>
<tr>
<td>213</td>
<td>5' CAGCGAACTA 3'</td>
</tr>
<tr>
<td>214</td>
<td>5' CATGTGCTTG 3’</td>
</tr>
<tr>
<td>215</td>
<td>5' TCACACGTGC 3’</td>
</tr>
<tr>
<td>216</td>
<td>5' CATAGACTCC 3’</td>
</tr>
<tr>
<td>217</td>
<td>5' ACAGGTAGAC 3’</td>
</tr>
<tr>
<td>218</td>
<td>5' CTCAGCCCAG 3’</td>
</tr>
<tr>
<td>219</td>
<td>5' GTGACCTCAG 3’</td>
</tr>
<tr>
<td>220</td>
<td>5' GTCGATGTCG 3’</td>
</tr>
</tbody>
</table>
PCR Assay

A 2 µl aliquot of Ba1 nematode DNA was added to a 200 µl PCR reaction tube. One µl of 10 µM random primers (reverse and forward primers) (Protech Technology Enterprise Co, Taiwan) (Table 2.1), 10 µl GoTaq® Hot Start Green 2X Master Mix (Promega, USA) and 7µl distilled water were placed in the tube and mixed thoroughly. A 5-minute 95°C activation step was used to eliminate non-specific DNA binding such as primer-dimers and mis-primed products. The thermocycling conditions were denaturation at 94°C for 5 minutes and 35 cycles of amplification (94°C for 30 seconds; 37°C for 30 seconds; 72°C for 30 seconds), followed by a final extension at 72°C for 7 minutes. The PCR product (10 µl) was loaded onto a 1% agarose gel and electrophoresed at 80 volts for 35 minutes. Size markers Gen100 (GeneMark, Taiwan) were co-electrophoresed with the DNA samples. The gels was stained with ethidium bromide for 30 minutes and then de-stained for 10 minutes to enable fluorescent visualization of the DNA fragments under UV light.

Ligation and Transformation

The amplified products were then cloned following the protocol of pGEM-T Vector System Kit (Promega, USA). Briefly a 5 µl 2 X Rapid Ligation buffer, 1 µl pGEM-T vector, 3 µl of PCR product amplified by random primers, 1 µl T₄ DNA Ligase (3 U/µl) and 1µl
distilled water were mixed thoroughly and incubated at 25°C for 1 hour, followed by overnight incubation at 4 °C. A 100 μl volume of competent cells was placed on ice for 5 minutes, and then added to 5 μl of the ligation mixture. The cells and ligation mix were vortexed for 1 second, and then placed on ice for 20 minutes. The solution was heat shocked by placing the tube in a 42°C water bath for 45 seconds followed by 2 minutes on ice. LB (Lysogeny broth, Promega) liquid medium (900 μl) was added in the tube, which was incubated for 1 hour at 37 °C. The tube was centrifuged for 10 minutes at 3,000 × g. The supernatant was discarded and the pellet resuspended in 100 μl LB medium. The resuspended pellet was plated on LB medium containing 50μg/ml ampicillin, 4 μl 200 mg/ml IPTG, 40 μl 20 mg/ml X-Gal. Plates were incubated 14-16 hour at 37 °C. White colonies were selected and transferred to a tube containing 2 ml LB medium with 50 μg/ml ampicillin. The tubes were incubated 14-16 hour at 37 °C.

**Plasmid Purification**

A Molecular Biology Tools Plasmid Miniprep Purification kit (GeneMark, Gmbiolab Co., Ltd, Taiwan) was used to extract plasmid DNA from the cultures. Bacterial cultures were centrifuged 2 minutes at 14,000 × g and the supernatant discarded. The pellet was resuspended in 200 μl of solution I. A 200 μl aliquot of solution II was added and the tube
shaken 5 times. Additionally, 200 μl of solution III was added and the tube shaken 5 times again. The mixture was centrifuged 5 minutes at 14,000 × g and the supernatant transferred to a spin column arranged on a collection tube and was centrifuged for 1 minute at 12,000 × g and the collection tube discarded. Washing buffer (700 μl) was added to the column which was again centrifuged for 1 minute at 14,000 × g. The collected fluid was discarded and the washing step repeated. The spin column was centrifuged for 3 minutes at 10,000 × g. The column was transferred to a clean 1.5 ml eppendorf tube and air dried for 1 hour. A 20-40 μl aliquot of distilled water was added to the tube which was incubated at 25°C for 3 minutes then centrifuged for 3 minutes at 14,000 × g to elute plasmid DNA.

A PCR assay was used to confirm the insertion of plasmid DNA. In each PCR tube, 1 μl of plasmid DNA was added as template along with 10 μl GoTaq® Hot Start Green 2X Master Mix (Promega, USA) and 1 μl each of 10 μM M13 Forward and Reverse primers (Tabel 2.3). Distilled water (7 μl) was added to make a final volume of 20 μl. The thermocycling conditions were denaturation at 94°C for 5 minutes, 30 cycles of amplification (94°C for 30 seconds; 50°C for 30 seconds; 72°C for 1 minute) followed by a final extension at 72°C for 5 minutes.
Table 2.3 PCR primers candidates used in nematode guild Bα1 universal qPCR primer development

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sense/antisense (+/-)</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nematode-11F</td>
<td>-</td>
<td>5' CAGATCGCCTCGATGCCC 3'</td>
</tr>
<tr>
<td>Nematode-11R</td>
<td>+</td>
<td>5' GCAGGGGAGGGTCAGCTT 3'</td>
</tr>
<tr>
<td>Nematode-17F</td>
<td>+</td>
<td>5' CGCACTATGAATGAGAAGATG 3'</td>
</tr>
<tr>
<td>Nematode-17R</td>
<td>-</td>
<td>5' TAGACATTTCAGATCAAACAACCTT 3'</td>
</tr>
<tr>
<td>Nematode-181F</td>
<td>+</td>
<td>5' TCACGTGCTCTTTAGAACATC 3'</td>
</tr>
<tr>
<td>Nematode-181R</td>
<td>-</td>
<td>5' CTGTGAATTCAAGAGATAGAAG 3'</td>
</tr>
<tr>
<td>Nematode-182F</td>
<td>-</td>
<td>5' GATCAGGGCTGATCAAAGGGT 3'</td>
</tr>
<tr>
<td>Nematode-182R</td>
<td>+</td>
<td>5' AGCTAACCAGGTAATCAACAGT 3'</td>
</tr>
<tr>
<td>Nematode-191F</td>
<td>-</td>
<td>5' GGTGATGAGAAGCGGACAAA 3'</td>
</tr>
<tr>
<td>Nematode-191R</td>
<td>-</td>
<td>5' TCTTTACTCTATACCTCTCTCC 3'</td>
</tr>
<tr>
<td>Nematode-192F</td>
<td>+</td>
<td>5' GCAGTATAGTAGTCGAAGTAG 3'</td>
</tr>
<tr>
<td>Nematode-192R</td>
<td>-</td>
<td>5' CTTTTGAGGGTTCTGGAAATA 3'</td>
</tr>
<tr>
<td>M13-F</td>
<td>+</td>
<td>5' GTTTTCCCAGTCAGGAC 3'</td>
</tr>
<tr>
<td>M13-R</td>
<td>-</td>
<td>5' CAGGAAACAGCTATGAC 3'</td>
</tr>
</tbody>
</table>

*a Nucleotide at degenerate positions are represented by a signal letter code; M = A, C*
The PCR product (5 µl) was loaded onto a 1% agarose gel and electrophoresed at 80 volts for 35 minutes. Size markers Gen100 (GeneMark, Taiwan) were co-electrophoresed with the DNA samples. The gels were stained with ethidium bromide for 30 minutes and then de-stained for 10 minutes to enable fluorescent visualization of the DNA fragments under UV light.

**Sequencing Method**

The random primer PCR products from previous PCR assay and cloning method were sequenced by Mission Biotechnology Company by using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM™ 3730 DNA Sequence (PerkinElmer, USA). The sequences obtained were saved as a *txt files and aligned with the GenBank of NCBI database using the BLASTn function. Alignment results were used as reference to design Ba1 universal primer sets (Table 2.3). Primer length ranged from 20-25 mer and PCR amplicon sized ranged from 100 to 200 bp. Primer sets were tested by Ba1 and Ba2 nematode DNA. Following PCR methods outlined previously.

**Ba2:** The representative of Ba2 18S rDNA sequence sets (Table 2.1) were collected from GenBank of NCBI and aligned by using Clustal W software. A ~200 bp conserved region of Ba2 was defined and used to design universal primer sets.
**F2:** Aphelenchoidea and Aphelenchoididae 18S rDNA sequences (Table 2.1) were collected from GenBank of NCBI and aligned by using Clustal W software. Universal qPCR primer sets for three major genera--*Aphelenchus, Filenchus* and *Aphelenchodies* were designed from two conserved region (~200 bp and ~100 bp).

**Om4, Om5 and P5:** The representative taxon 18S rDNA sequences were aligned and a ~200 bp conserved region was determined (Table 2.1).

**P4:** Eighteen sets of Mononchidae 18S rDNA sequence were collected from GenBank of NCBI and aligned by using Clustal W software. A ~200 bp conserved region was selected and use to design qPCR primer set.

After determining a conserved region that is < 200 bp long (as qPCR amplicons should be restricted to 50-200 bp) and identified the qPCR primer location candidates, primer candidates were analyzed for secondary structure, melting temperature, stem loop, self-hybridizing and G/C content by TIB MolBiol (Berlin, Germany) and universal qPCR primer sets were determined.

**Confirmation of qPCR Primer Sets**

After development of universal qPCR primer sets, nematode DNA was used as template to
each test the primer efficiency. A 1 µl nematode DNA has been extracted from exact nematodes (either Ba1, Ba2, F2, Om4/Om5/P5 or P4) was added as template to a 10 µl GoTaq® Hot Start Green 2X Master Mix (Promega, USA) with 1 µl each of 10 µM corresponding universal qPCR forward and reverse primers which (Table 2.3), 7 µl of distilled water was added to make final volume 20 µl. The thermocycling conditions were denaturation at 94°C for 5 minutes followed by 30 cycles of amplification (94°C for 30 seconds; 50°C for 30 seconds; 72°C for 1 min), with a final extension at 72°C for 5 minutes. The PCR product (5 µl) was loaded onto a 1% agarose gel and electrophoresed at 80 volts for 35 minutes. Size markers Gen50 (GeneMark, Taiwan) were co-electrophoresed with the DNA samples. The gels were stained with ethidium bromide for 30 minutes and then de-stained for 10 minutes to enable fluorescent visualization of the DNA fragments under UV light.

**Results**

Universal qPCR primers were developed using Clustal W software to align sets of 18S rDNA sequences from representative taxon of nematode guilds. However, none of the conserved regions found from the alignment results of Clustal W could differentiate Ba1 nematodes from Ba2 nematodes. To overcome the insufficiency of the Genbank database, universal qPCR primers for the Ba1 nematode taxon were developed using the random
priming PCR method. Random priming gave several different amplicons. Random primer 211, 217 and 218 produced a 100-bp, 350-bp and 300-bp amplicon, respectively. Random primer 219 gave 4 amplicons as 150 bp, 250 bp, 300 bp and 400 bp (Fig. 2.1). Because the vector primer sets added 200 bp more to the insert, plasmid 11-1 to 11-5 produced a 350-bp PCR amplicon. Plasmids 17-1 to 17-5 produced a 550-bp PCR amplicon. Plasmids 18-1 to 18-5 produced a 500-bp PCR amplicon and plasmid 19-1 to 19-5 produced a 600-bp PCR amplicon (Fig. 2.2). Ba1 specific primers tested against Ba1 and Ba2 DNA were not always specific. Primers Nematode-11, Nematode-182, Nematode-192, Nematode-181 and Nematode-191 gave 100-bp, 150-bp, 200-bp, 250-bp and 350-bp amplicons respectively with Ba1 and Ba2 nematode DNA. However, Nematode-17 only produced a 300-bp amplicon with Ba2 template. The random priming PCR method failed to provide a universal qPCR primer set for Ba1 nematodes (Fig. 2.3).
Figure 2.1 PCR of Ba1 nematode DNA with random primer No.211 to 220
lane M: 100 bp-3 kb DNA markers (GeneMark, Taiwan).
Lane 1: random primer of No.211
Lane 2: random primer of No.212
Lane 3: random primer of No.213
Lane 4: random primer of No.214
Lane 5: random primer of No.215
Lane 6: random primer of No.216
Lane 7: random primer of No.217
Lane 8: random primer of No.218
Lane 9: random primer of No.219
Lane 10: random primer of No.220
Figure 2.2 Electrophoretic analysis of the PCR products amplified from transformed plasmid DNA with M13 Forward and Reverse primers in 1.5% agarose gels.

lane M: 100 bp-3 kb DNA markers (GeneMark, Taiwan)

lane 1: plasmid 11-1 PCR product  lane 11: plasmid 18-1 PCR product
lane 2: plasmid 11-2 PCR product  lane 12: plasmid 18-2 PCR product
lane 3: plasmid 11-3 PCR product  lane 13: plasmid 18-3 PCR product
lane 4: plasmid 11-4 PCR product  lane 14: plasmid 18-4 PCR product
lane 5: plasmid 11-5 PCR product  lane 15: plasmid 18-5 PCR product
lane 6: plasmid 17-1 PCR product  lane 16: plasmid 19-1 PCR product
lane 7: plasmid 17-2 PCR product  lane 17: plasmid 19-2 PCR product
lane 8: plasmid 17-3 PCR product  lane 18: plasmid 19-3 PCR product
lane 9: plasmid 17-4 PCR product  lane 19: plasmid 19-4 PCR product
lane 10: plasmid 17-5 PCR product  lane 20: plasmid 19-5 PCR product
Figure 2.3 Electrophoretic analysis of the PCR products with Ba1 and Ba2 DNA amplified using specific primers designed from cloned DNA sequences of Ba1 DNA with random primers in 1.5% agarose gel.
LaneM : 100-3,000bp DNA markers (Gene Mark, Taiwan)
Lane 1: Ba1-No. 11 primer
Lane 2: Ba1-No. 182 primer
Lane 3: Ba1-No. 192 primer
Lane 4: Ba1-No. 181 primer
Lane 5: Ba1-No. 17 primer
Lane 6: Ba1-No. 19-1 primer
Lane 7: Ba2-No. 11 primer
Lane 8: Ba2-No. 182 primer
Lane 9: Ba2-No. 192 primer
Lane 10: Ba2-No. 181 primer
Lane 11: Ba2-No. 17 primer
Lane 12: Ba2-No. 19-1 primer
Primer efficiency for Ba2, F2, Om4, Om5, P4, P5 was showed clearly (Fig. 2.4). Each qPCR primer set successfully amplified the specific amplicon expected. Lane1 was the Ba2 DNA/Ba2 universal qPCR primer set which was estimated to be 159 bp. Lane2 was the F2 DNA/F2-1 universal qPCR primer set (*Aphelenchus/Filenchus*) which was estimated to be 153 bp (Table 2.4) and amplified a ~150 bp product. The F2 DNA/F2-2 universal qPCR primer set (*Aphelenchoides*) in lane 3 was estimated to give a 85 bp amplicon which it did. The Om4, Om5, and P5 DNA/Om4, Om5, P5 universal qPCR primer set was estimated to give a 139 bp and amplified a ~140 bp product. The P4 DNA/P4 universal qPCR primer set was estimated to be a 196 bp amplicon gave a product of this size. Universal qPCR primers for Ba2, F2, Om4, Om5, P4, P5 was readily identified using conserved regions of the 18S rDNA. However, in F2, *Aphelenchus* and *Filenchus* showed more similarity to each other compared to *Aphelenchoides*. In order to detect the abundance of these three major genera of Aphelenchoidea, two sets of universal primers (one for *Aphelenchus* and *Filenchus*, the other one for *Aphelenchoides*) were developed. Om4, Om5 and P5 have high homology within the conserved 18S rDNA region. One universal qPCR primer set was therefore developed for Om4/Om5/P5. A single primer set was sufficient to differentiate P4 nematodes from other guilds (Table 2.4).
Figure 2.4 Electrophoretic analysis of the PCR products with Ba2 F2, Om4, Om5, P4, and P5 DNA amplified using universal qPCR primer sets specifically to above guilds in 1.5% agarose gel.

Lane M: 50bp -500bp Gen50 DNA Marker (GeneMark, Taiwan)
Lane 1: Ba2 DNA/Ba2 universal qPCR primer set
Lane 2: F2 DNA/F2 universal qPCR primer set (*Aphelenchus/ Filenchus*)
Lane 3: F2 DNA/F2 universal qPCR primer set (*Aphelenchodies*)
Lane 4: Om4, Om5, and P5 DNA/Om4, Om5, P5 universal qPCR primer set
Lane 5: P4 DNA/P4 universal qPCR primer set
Table 2. 4 Universal qPCR primer sets developed for nematode guilds Ba2 F2, Om4, Om5, P4, and P5.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>qPCR amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-F</td>
<td>GTAGCCTTCACTGGTTGCAG</td>
<td>159</td>
</tr>
<tr>
<td>Ba2-R</td>
<td>CCTTGTTAACCATTATCTCAGTCCAC</td>
<td></td>
</tr>
<tr>
<td>F2-1-F</td>
<td>GGYGTTCAGCCGCACGAG</td>
<td>153</td>
</tr>
<tr>
<td>F2-1-R</td>
<td>TTCCGATCCCAATCACG</td>
<td></td>
</tr>
<tr>
<td>F2-2-F</td>
<td>CACGCAGGATTACTTTGAACG</td>
<td>85</td>
</tr>
<tr>
<td>F2-2-R</td>
<td>GCAACCTTGTCCATATTCCAT</td>
<td></td>
</tr>
<tr>
<td>Om4/Om5/P5-F</td>
<td>TCGCACTTCTTAGGGGACAA</td>
<td>139</td>
</tr>
<tr>
<td>Om4/Om5/P5-R</td>
<td>GGTTCTTCCGAACTAGGTATAACG</td>
<td></td>
</tr>
<tr>
<td>P4-F</td>
<td>CGAGACTCTAGCCTATTAAATAGACA</td>
<td>196</td>
</tr>
<tr>
<td>P4-R</td>
<td>TTTCGTGTTTACACATTCTTAC</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Universal qPCR primer sets specific for Ba (but not Ba1 and Ba2 separately), F2, and Om4/Om5/P5 in conjunction (but not individually), and P4 were determined using GenBank. Although the GenBank is particularly biased to most plant- and animal-pathogenic nematodes (Powers, 2004; Blok, 2005), the information provided is sufficient to develop qPCR primer sets which can be applied to nematode community analysis.

The development of a Ba1 nematode universal qPCR primer set required a different strategy than the other guilds due to the high similarity between Ba1 and Ba2. The DNA sequences are conserved in the region and insufficient sequence information is available in GenBank in NCBI to determine the needed differences that presumably exist (Powers, 2004; Blok, 2005). To determine a critical sequence fragment specifically to Ba1 nematodes, random primers were used to amplify Ba1 under a low primer annealing temperature ($37^\circ C$) (Feinberg, 1993). Separation still revealed difficult as 5 of 6 Ba1 universal qPCR primer set candidates could not differentiate among Ba1 and Ba2 nematodes. Moreover, the one Ba1 universal qPCR primer set with specificity, reacted with Ba2 nematode DNA after multiple repeats. Since Ba1 universal qPCR primer sets were designed from the cloned Ba1 nematode DNA, the failure to amplify Ba1 nematode DNA might be because the random priming PCR
method annealing temperature was too low and unspecifically annealed to Ba1 DNA. Although the amplicons were cloned and sequenced, sequences located at primer binding sites might not reveal the actual sequences. Thus, a Ba1 nematode universal qPCR primer set could not be developed. Nematode community analysis parameters can be adjusted for the absence of Ba1 nematode abundance. The universal qPCR primer set for Ba2 should partially indicate the bacterial decomposition in the soil when investigating different nematode parameters and indices. In future, Ba1 universal qPCR primer might be obtained by conducting more random primers to search for a Ba1 specific region, or design a universal qPCR primer for Ba1/Ba2 guilds together, and subtracting Ba2 data to have Ba1 data.

The F2 nematode universal qPCR primer sets were successfully developed using the same strategy as for the Ba2 guild. During the alignment of Aphelenchidae nematodes, high homology was observed between conserved regions of *Aphelenchus* and *Filenchus*. Furthermore, *Aphelenchodies* have been phylogenetically changed to family Aphelenchoididae (De Ley, 2002). Thus, one primer set was developed using *Aphelenchus* and *Filenchus*, and another primer set using *Aphelenchodies* sequence data. In the F2 guild, two primers are needed for qPCR. The abundance obtained from the reaction of both primer sets must be combined to calculate nematode parameters and nematode indices.
Having one set of qPCR primer for Om4, Om5 and P5 nematodes to detect the abundances of these nematodes that are mostly in the dorylaimid group simplify the molecular protocol for percentage of omnivores and predatory nematode calculation. However, an additional primer set is still required for the P4 nematodes, which is dominated by the mononchids. However, further primer set development is still required to separate Om4, Om5 and P5. To calculate SI (structure index), weighted constants are multiplied to each guild at different weight: 0.8 for \( cp-2 \); 1.8 for \( cp-3 \); 3.2 for \( cp-4 \); 5 for \( cp-5 \). Thus, abundance of nematode guilds of Om4, and P4 need to be separated from Om5 and P5. It is realized that it might not be feasibly calculate all nematode community indices as suggested by Neher (2001) and Ferris et al., (2001). For the objective of this research on developing molecular tools for comparing soil health conditions among ecosystems, we are proposing to just standardize one weight constant, i.e. 3.2 for Om4, Om5 and P5. The justification is that Om4 nematodes are usually more abundant than Om5 and P5 nematodes, and our objective is to compare among ecosystem with the same nematode community index calculation.

When searching for the qPCR aiming region, alignment results showed that sequences were conserved within same feeding group of nematodes, therefore there might be a relationship exists between nematode feeding habit and morphology (Yeates, 1993; Bongers,
Since feeding habits and morphology of nematodes are controlled by gene expression (Gorokhova, 2002; Waite, 2003; Powers, 2004; Blok, 2005), a relationship is also logically correlated with sequence homology. The 18S rDNA sequences alignment showed that guild/feeding habit/morphology might correlate with sequence homology.

In conclusion, universal qPCR primers for Ba2, F2, P4, and Om4/Om5/P5 in conjunction were developed from this research. Challenge remained to develop a specific but universal primer for Ba1, and to separate Om4 from Om5 and P5 if calculation of EI, SI, and CI are needed to compare nematode communities from different ecosystem. Tentatively, this research provides us with an option to obtain abundance of Ba1 by designing a universal qPCR primer set for Ba1/Ba2, and substrating Ba2 from Ba1/Ba2 data, thus would allow us to calculate EI and CI. In terms of SI, while we could not conform to the calculation of SI as published by Ferris et al. (2001), we are suggesting a modified SI using 3.2 as the weight constant for Om4,Om5 and P5 assuming that nematodes with c-p value 4 are much more abundant than those with c-p value 5.
Literature Cited


Chapter 3

Determine ability of universal qPCR primer sets to indicate for nematode guild Ba1, Ba2, F2, P4, P5, Om4, and Om5

Five sets of universal qPCR primer were developed using 18S rDNA sequence information from GenBank at the NCBI previously. In this chapter, these universal qPCR primers were evaluated employing two approaches: BLAST at the NCBI and PCR method. Utilizing Genbank BLAST out universal qPCR primer sets to confirm the primer specificity to the desired representative taxon of each guild, yet the alignment between primer sets and other nematode functional guilds needs to be known. The qPCR primer sets were ultimately applied to soil DNA sample that had a mixture of templates. The overall nematode community is the target of the soil food web of soil health research, the specificity of qPCR primers should be of the most concern. Thus, the objective of this chapter was to confirm the specificity of qPCR primer sets that will ultimately be applied to soil DNA samples, which compose of a mixture of different nematode guilds. The second objective of this research was to further confirmed these PCR amplicons were the nematode guilds desired, DNA sequences were determined by sequencing method.
Material and Method

On-line Database- To evaluate primer specificity to nematode guilds (Table 2.4), primers were aligned to the entire Nematoda database at NCBI (National Center of Biotechnology Information). The alignment was conducted using the ‘nucleotide blast’ (BLASTn). The primer sets were tested for specificity within the trophic group/ c-p value. Table data were generated showing the alignment result between five universal primer sets and GenBank.

Artificial Nematode Sample- To evaluate specificity in situ, synthesized primer sets were tested using an artificial nematode sample. Nematode samples were constructed using known number and genera of nematodes. Sample included nematodes from Ba1, Ba2, F2, Om4, Om5, P4, and P5 guilds. One nematode from each guild was collected from soil collected from Oahu, Hawaii. Nematodes were added to a 1.5ml eppendorf with 200 μl distilled water and stored at -20°C for DNA extraction and PCR assay.

DNA from artificial nematode sample was extracted using the PureLink™ DNA Extraction Kit (Invitrogen) with a protocol modified with bead-beating and heating steps (Chapter 2, Donn, 2008). For the PCR, 2 μl of extracted nematode DNA was added to a 200 μl PCR reaction tube, 1 μl 10 μM Ba2, F2, Om4, Om5, P4, P5 universal qPCR primers (reverse and forward primers) (Table 2.4) as the PCR protocol described in chapter 2. The amplified
products were then cloned using pGEM-T Vector System Kit (Promega, USA) and *Ecoli* transformation to amplify plasmids in bacteria competent cells following the protocol in chapter 2.

A Molecular Biology Tool Plasmid Miniprep Purification kit (GeneMark, Gmbiolab Co., Ltd, Taiwan) was used to extract plasmid DNA from the cultures followed by a PCR assay used to confirm the insertion of plasmid DNA using M13 forward and reverse primers (Table 2.4) as the protocol described in chapter 2. The PCR products from previous experiments of PCR assay, ligation, transformation and plasmid miniprep were then PCR confirmation by sequencing (Mission Biotechnology Company) using ABI PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction kit and ABI PRISMTM 3730 DNA Sequence (PerkinElmer, USA). The sequences obtained were saved as *.txt* files and align with the GenBank of NCBI database using BLASTn function.

**Results**

The alignment results showed that the qPCR primer sets (both forward and reverse) for *Ba*2, *F*2, *Om4/Om5/P5*, and *P*4 were 100% identical to the nematode guilds for which they were designed. The primers did not have identity to other nematode guilds. The reverse
primer for F2 aligned to *Anguina* with a 100% identity (Table 3.2).

The qPCR primer sets have been examined and successfully tested in a PCR assay using individual nematode DNA as template (Chapter 2, Wang, 2011). The current experiment using artificial nematode samples (composed of different nematode guilds) to mimic agricultural samples further verified the specificity of qPCR primer sets listed in Table 2.4. Each qPCR primer set successfully amplified the amplicon expected, however Ba2, F2-1 and P4 have showed extra faint amplicons (Fig 3.1). The DNA mixture / Ba2 universal qPCR primer set was estimated to be 159 bp and gave a amplicon ~160 bp (Fig 3.1). The DNA mixture/F2-1 universal qPCR primer set (*Aphelenchus/Filenchus*) was estimated to be 153 bp and produced a band ~150 bp (Fig 3.1). The DNA mixture/F2-2 universal qPCR primer set (*Aphelenchodies*) was estimated to be 85 bp and the product was ~85 bp (Fig 3.1). The DNA mixture/Om4, Om5, P5 universal qPCR primer set was estimated to be a 139 bp amplicon and it gave a ~140 bp product (Fig 3.1).

The DNA mixture/P4 universal qPCR primer set was estimated to amplify a 196 bp amplicon, and the product was a ~200 bp band. The qPCR primer sets developed were all specific to the nematode guilds they were designed to detect, and amplified the appropriate sized amplicon (Table 2.4). The mixed template demonstrated that these primer sets were
able to detect and properly identify nematode guilds in a field sample.

Table 3.1 Alignment of qPCR primer sets developed for nematode guilds Ba2, F2, Om4/Om5/P5, and P4.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Percentage of Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-F</td>
<td>GTAGCCTTCCTGTTGCGG</td>
<td>100% to Ba2</td>
</tr>
<tr>
<td>Ba2-R</td>
<td>CCTTGTTAACCATTATCTCAGTCCAC</td>
<td>100% to Ba2</td>
</tr>
<tr>
<td>F2-1-F</td>
<td>GGYGTTCAGCGCAACGAG</td>
<td>100% to F2- <em>Aphelenchus</em>/<em>Filenchus</em></td>
</tr>
<tr>
<td>F2-1-R</td>
<td>TTCCGATCCCAATCAGG</td>
<td>100% to F2- <em>Aphelenchoides</em></td>
</tr>
<tr>
<td>F2-2-F</td>
<td>CACGCAGGATTACTTGAACG</td>
<td>100% to F2- <em>Aphelenchoides</em></td>
</tr>
<tr>
<td>F2-2-R</td>
<td>GCAACCTTGTTCCATTATCCAT</td>
<td>100% to F2- <em>Aphelenchoides</em></td>
</tr>
<tr>
<td>Om4/Om5/P5-F</td>
<td>TCGCACTTCTTAGGAGGACAA</td>
<td>100% to Om4/Om5/P5</td>
</tr>
<tr>
<td>Om4/Om5/P5-R</td>
<td>GGTTCCTCCGAATAGGTTATAACG</td>
<td>100% to Om4/Om5/P5</td>
</tr>
<tr>
<td>P4-F</td>
<td>CGAGACTCTAGGCTTAAATAGCA</td>
<td>100% to P4</td>
</tr>
<tr>
<td>P4-R</td>
<td>TTTCGTGTTTACCAATTCTTAC</td>
<td>100% to P4</td>
</tr>
</tbody>
</table>
Figure 3.1 Electrophoretic analysis of the PCR products with nematode guild Ba2 F2, Om4, Om5, P4, and P5 DNA amplified using universal qPCR primer sets specific to the guilds in 1.5% agarose gel.

Lane M: 50bp -500bp Gen50 DNA Marker (GeneMark, Taiwan)
Lane 1: mixture DNA/Ba2 universal qPCR primer set
Lane 2: mixture DNA/F2-1 universal qPCR primer set (Aphelenchus/ Filenchus)
Lane 3: mixture DNA/F2-2 universal qPCR primer set (Aphelenchodies)
Lane 4: mixture DNA/Om4, Om5, P5 universal qPCR primer set
Lane 5: mixture DNA/P4 universal qPCR primer set
To further confirmed these PCR amplicons were the nematode guilds desired, DNA sequences were determined by sequencing. PCR amplicons were cloned using pGEM-T vector system and confirmed using PCR with M13 F/R vector primer set, the result was 180 bp larger (vector sequences) PCR amplicon. Therefore the plasmid had the correct Ba2 qPCR insert should have 159 bp (qPCR amplicon) plus 180 bp (vector sequences) for ~339 bp, and F2 (Aphelenchus/Filenchus), F2 (Aphelenchodie), Om4/Om5/P5 and P4 were 333 bp, 265 bp, 319 bp, and 376 bp amplicons, respectively. Plasmids with no qPCR amplicon insert should reveal a 180 bp PCR amplicon.

Plasmids Ba2-2, 4, 5, 7, 8, 10 showed a ~339 bp PCR product which is the qPCR amplicon plus vector sequences (Fig. 3.2) while Ba2-1, 3, 9 showed a 180 bp product that were empty vectors. Moreover, Ba2-6 showed multiple bands of 200 bp, 300 bp and 400 bp, which might be the misamplification of the empty vectors (Fig.3.2).

Plasmid F2 (Aphelenchus/Filenchus)-1, 2, 4, 5, 6, 8, 9, 10 had the expected PCR amplicon size of 333 bp, whereas F2 (Aphelenchus/Filenchus)-3, 7 showed a 180 bp vector amplicon from empty vector (Fig 3.2-3). F2 (Aphelenchodie)-1, 2, 4, 6 showed the expected PCR amplicon ~265 bp, whereas F2 (Aphelenchodie)- 3, 5, 8, 9 had showed a 180 bp vector
amplicon produced from the empty vector. F2 (*Aphelenchodies*)- 7, 10 also had multiple bands of 150 bp/ 250 bp/ 350bp and 150bp/ 200bp/ 250bp/ 350bp/ 1.5 kb that might express also misamplification of an empty vector (Fig 3.3).

Om4/Om5/P5- 1, 5, 6, 8, 9 had a 339 bp PCR amplicon, revealing the qPCR amplicon of Om4/Om5/P5 (159 bp) plus 180 bp vector sequences Om4/Om5/P5- 3, 4, 7 had a 180 bp vector amplicon from empty vector. Moreover, Om4/Om5/P5- 2 had no PCR amplicon that might be due to no plasmid being extracted. Om4/Om5/P5- 10 had multiple bands of 200bp/ 340bp/450bp that might be the mis-amplication of empty vector (Fig 3.4). While P4- 1, 2, 4, 6, 7, 8, 9, 10 gave a 400 bp PCR product as expected for P4 qPCR product size plus the 180 bp vector sequences. P4- 3 and 5 had multiple bands of 100 bp/ 150 bp/ 200bp/ 250bp that might be the misamplification of empty vector (Fig 3.4).

The plasmids- Ba2-2, 5, 10; F2-*Aphelenchus/Filenchus*- 1, 5, 8; F2-*Aphelenchodies*- 1, 2, 4; Om4/Om5/P5- 5, 6, 8; P4- 1, 2, 4. All qPCR amplicon sequences showed 100% identity to the representative taxon that were selected as the sources to develop qPCR primer sets. Few other taxon (within same nematode guild) which the primer was not designed from were also detected (Table 3.2-3).
Figure 3.2 Electrophoretic analysis of the PCR products amplified from transformed plasmid DNA with M13 Forward and Reverse primers in 1.5% agarose gels.

lane M1: Gen100, 100 bp-3 kb DNA markers (GeneMark, Taiwan)
lane M2: Gen50, 50 bp- 500 bp DNA markers (GeneMark, Taiwan)
lane 1: plasmid Ba2-1
lane 2: plasmid Ba2-2
lane 3: plasmid Ba2-3
lane 4: plasmid Ba2-4
lane 5: plasmid Ba2-5
lane 6: plasmid Ba2-6
lane 7: plasmid Ba2-7
lane 8: plasmid Ba2-8
lane 9: plasmid Ba2-9
lane 10: plasmid Ba2-10
lane 11: plasmid F2(Aphelenchus/Filenchus)-1
lane 12: plasmid F2(Aphelenchus/Filenchus)-2
lane 13: plasmid F2(Aphelenchus/Filenchus)-3
lane 14: plasmid F2(Aphelenchus/Filenchus)-4
lane 15: plasmid F2(Aphelenchus/Filenchus)-5
Figure 3.3 Electrophoretic analysis of the PCR products amplified from transformed plasmid DNA with M13 Forward and Reverse primers in 1.5% agarose gels.

lane M1: Gen100, 100 bp-3 kb DNA markers (GeneMark, Taiwan)
lane M2: Gen50, 50 bp-500 bp DNA markers (GeneMark, Taiwan)
lane 1: plasmid \( F_2(\text{Aphelenchus/Filenchus})-6 \)
lane 2: plasmid \( F_2(\text{Aphelenchus/Filenchus})-7 \)
lane 3: plasmid \( F_2(\text{Aphelenchus/Filenchus})-8 \)
lane 4: plasmid \( F_2(\text{Aphelenchus/Filenchus})-9 \)
lane 5: plasmid \( F_2(\text{Aphelenchus/Filenchus})-10 \)
lane 6: plasmid \( F_2(\text{Aphelenchodie})-1 \)
lane 7: plasmid \( F_2(\text{Aphelenchodie})-2 \)
lane 8: plasmid \( F_2(\text{Aphelenchodie})-3 \)
lane 9: plasmid \( F_2(\text{Aphelenchodie})-4 \)
lane 10: plasmid \( F_2(\text{Aphelenchodie})-5 \)
lane 11: plasmid \( F_2(\text{Aphelenchodie})-6 \)
lane 12: plasmid \( F_2(\text{Aphelenchodie})-7 \)
lane 13: plasmid \( F_2(\text{Aphelenchodie})-8 \)
lane 14: plasmid \( F_2(\text{Aphelenchodie})-9 \)
lane 15: plasmid \( F_2(\text{Aphelenchodie})-10 \)
Figure 3.4 Electrophoretic analysis of the PCR products amplified from transformed plasmid DNA with M13 Forward and Reverse primers in 1.5% agarose gels.

lane M1: Gen100, 100 bp-3 kb DNA markers (GeneMark, Taiwan)
lane M2: Gen50, 50 bp-500 bp DNA markers (GeneMark, Taiwan)

lane 1: plasmid Om4/Om5/P5-1
lane 2: plasmid Om4/Om5/P5-2
lane 3: plasmid Om4/Om5/P5-3
lane 4: plasmid Om4/Om5/P5-4
lane 5: plasmid Om4/Om5/P5-5
lane 6: plasmid Om4/Om5/P5-6
lane 7: plasmid Om4/Om5/P5-7
lane 8: plasmid Om4/Om5/P5-8
lane 9: plasmid Om4/Om5/P5-9
lane 10: plasmid Om4/Om5/P5-10
lane 11: plasmid P4-1
lane 12: plasmid P4-2
lane 13: plasmid P4-3
lane 14: plasmid P4-4
lane 15: plasmid P4-5
lane 16: plasmid P4-6
lane 17: plasmid P4-7
lane 18: plasmid P4-8
lane 19: plasmid P4-9
lane 20: plasmid P4-10
<table>
<thead>
<tr>
<th>Nematode Guild</th>
<th>qPCR Amplicon</th>
<th>Sequencing Result</th>
</tr>
</thead>
</table>
| Ba2            | CACGCAATTCAGACCAAGATCCGAG 85 | 1 GGTGTTCAGCCGCACGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGG 61
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 CTGCACGCGCGCTACACTGGCGAAATCAGCGTGCTTCTCCTATCTCGAAAGAGACTGGTA 61 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 AACCGATGAAAATTTGCCGTGATTGGGATCGGAA 121 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 GTAGCCTTCACTGGTTGCGGCGGGTGGCTGGCGAGTTTACTTTGAGAAAATCAGAGTGCT 61 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 CAATACAGGCGTATCGCTTGAATGATTGTGCATGGAATAATAGAAAAGGATTTCGGTCCT 61 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 CTTTTATTGGTTTTGTGGACTGAGATAATGGTTAACAAGG 121 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 G6TTCGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 157 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 G6TTCGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 157 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 G6TTCGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 157 |
|                | CACGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 85 | 1 G6TTCGCAATTCAGACCAAGATCCGAGAATTGCAGGTTGC 157 |

Table 3.2: Sequences of qPCR amplicon of nematode guilds - Ba2, P2, O4m3, O4m4, Om7, and Pf.
Table 3.2 (continued) Sequences of qPCR amplicon of nematode guilds- Ba2, F2, Om4/Om5/ P5, and P4

<table>
<thead>
<tr>
<th>qPCR Amplicon</th>
<th>Sequencing Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>181</td>
<td>AVGAATTCATTAAACCAAA</td>
</tr>
<tr>
<td>121</td>
<td>TCCGACTCACTATAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGTTCGGAAGAACC</td>
</tr>
<tr>
<td>61</td>
<td>GACGAGCAGCTTCTAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>196</td>
<td>AAGAATTGGTAAACACGAAA</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGTTCGGAAGAACC</td>
</tr>
<tr>
<td>139</td>
<td>p4</td>
</tr>
<tr>
<td>61</td>
<td>GTGATGCCCTTAAGGCTACACTGAAAGAATCAGTGTGCGTT</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>139</td>
<td>Om4/Om5/</td>
</tr>
<tr>
<td>61</td>
<td>TCCGACTCACTATAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>196</td>
<td>AAGAATTGGTAAACACGAAA</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGCTGCACGAGATTGAGCAATAACAGG</td>
</tr>
<tr>
<td>121</td>
<td>ATACGACTCACTATAGTTCGGAAGAACC</td>
</tr>
<tr>
<td>139</td>
<td>p4</td>
</tr>
</tbody>
</table>

Table 3.2 (continued) Sequences of qPCR amplicon of nematode guilds- Ba2, F2, Om4/Om5/ P5, and p4
Table 3.3 Alignment of qPCR amplicon of nematode guilds- Ba2, F2, Om4/Om5/P5, and P4.

<table>
<thead>
<tr>
<th>qPCR amplicon</th>
<th>Percentage of Identity</th>
<th>Nematode Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2</td>
<td>100%</td>
<td>Ba2- *Eucephalobus, Acrobelesodies, Dicelis, Zelda, Cervidellus, Pseudoacrobeles, Chiloplacus, and Acrobeles</td>
</tr>
<tr>
<td>F2-1</td>
<td>100%</td>
<td>F2- <em>Aphelenchus, and Filenchus</em></td>
</tr>
<tr>
<td><em>Aphelenchus/Filenchus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2-2</td>
<td>100%</td>
<td>F2- <em>Aphelenchodies</em></td>
</tr>
<tr>
<td><em>Aphelenchodies</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Om4/Om5/P5</td>
<td>100%</td>
<td>Om5- <em>Aporcelaimellus and Paraxonchium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P5- <em>Paravulvus, Paractinolaimus and Nygolaimus</em></td>
</tr>
<tr>
<td>P4</td>
<td>100%</td>
<td>P4- <em>Prionchulus, Mylonchus and Mononchus</em></td>
</tr>
</tbody>
</table>
Discussion

Many tools can be applied to primer design and primer specificity examination. The primer BLAST software- Primer 3 provided by NCBI (Rozen, 2000), and MFE primer design software (Qu, 2009), as well as many biotechnology companies primer design/assessment tools are available. Proprietary software developed by biotechnology companies, TIB MolBiol (German) and Mission Biotech (Taiwan) are two examples. TIB MolBiol has been used to analyze several primer candidates developed for the Roche real time PCR instrument (Hardick, 2004).

qPCR primer sets were developed for Ba2, F2, Om4/Om5/ P5, and P4 guilds (Wang, 2011). These qPCR primer sets are specific and confirmed by BLASTn of the NCBI, PCR assay, and sequencing. The specificity of qPCR primer sets (forward and reverse) for Ba2, F2- *Aphelenchus/ Filenchus*, F2- *Aphelenchodies*, Om4/Om5/ P5 and P4 was 100% within and across nematode guilds. A PCR assay with a mixture of nematode guild DNA was also 100% specific for all qPCR primer sets. The PCR amplicon sequences revealed that all qPCR primer sets were able to amplify the specific 18S rDNA to which they were designed to detect. Nevertheless, we also need to discuss that some other guild not include in this dissertation but are also quite common in Hawaii as well as other places. For example P3
(Tripyla, Tobrillus); Ba3 (Prismatolaimus), and F3 (Diptherophora, Tylencholaimus, Leptonchus) which are also common in soil ecosystems.

In conclusion, the universal qPCR primer sets designed for nematode guilds Ba2, F2, Om4/Om5/ P5, and P4 have showed 100% identity to the targeted sequences of all the nematode genera in the same guild that are present in the NCBI data base, thus supporting their specificity on the targeted nematode guild. While there was limited nematode DNA sequence available in the NCBI Genbank. The results suggested that the universal qPCR primer sets developed are qualified to detect and quantify the targeted nematode guild, and these primer sets should be ready for use on environmental samples for nematode community analysis.
Literature Cited


Qu, W., Shen, Zhiyong, Zhao, Dongsheng, Yang, Yi, and Zhang, Chenggang, 2009. MFEprimer: multiple factor evaluation of the specificity of PCR primers. *Bioinformatics* 25, 276-278.


Chapter 4

Evaluate and compare real-time quantitative PCR (qPCR) and visual nematode identification for nematode soil community analysis

Assessment of soil health is one key component to evaluate the sustainability of an agricultural practice (Karlen, 1997; Doran, 2000). An assessment that utilizes soil nematode community analysis provides the depth and breadth needed to ascertain soil health. Nematode population composition reflects the soil nutrient status and its level of disturbance. Nematodes can be utilized as soil health indicators because of their abundance and ubiquitousness. Also nematodes have feeding behaviors and life strategies that transcend trophic levels, as well as interactions with other soil organisms. Nematodes are easy to sample and extract from the soil, and their morphology reflects their feeding behavior (Yeates, 1993).

For the last two decades, nematode ecologists had developed and strengthen nematode faunal analysis as a stringent tool to analyze soil health (Bongers, 1998; Neher, 1998; Ferris, 2001; Neher, 2001; Neher 2001). Traditionally, nematode community analysis has been conducted by visual identification. For nematode community analysis, identification of all nematodes to family, genus, or better yet the species level are needed (Bongers, 1998; Ferris, 2001).
reliance on visual morphological features observed by experts and the time required to make identifications are two main factors limiting the greater adoption of nematodes as soil health indicators. Use of molecular tools for nematode community analysis might overcome these limitations. Among many molecular methods reviewed in Chapter 1, qPCR assay is the only method that can achieve the function of identification and absolute-quantification of nematodes by taxon. In previous chapters, qPCR primer sets were developed for key nematode guilds to be used in nematode community analysis (Chapter 2, 3 and Wang, 2011). The next logical step is to verify the use of qPCR assay for nematode community analysis on environmental samples. Specific objective of this research is to compare the results of nematode community analysis using qPCR method with the traditional visual identification method in tandem by looking at four distinct soil ecosystems in Hawaii.

**Material and Methods**

**Nematode Collection and Extraction**

Soil samples were taken from a certified organic site at the Waimanalo University of Hawaii Experimental Station, Oahu, HI; a pineapple field with a *Crotalaria juncea* L. cover crop at the Whitmore University of Hawaii Experiment Station, Whitmore, Oahu, HI; a forest
site in University of Hawaii Lyon Arboretum, Oahu, HI, and from a beach site in Hanauma Bay Nature Park, Oahu, HI. Six soil cores from 20-cm deep were systematically collected from each area, composited into one sample, and transported to the laboratory. The soil was pored through a 1-cm inch screen. Nematodes were extracted from a 125-cm³ subsample by elutriation (Byrd, 1976) followed by centrifugal flotation (Jenkins, 1967). Visual identification and qPCR method each had one sub-sample from four samples. The nematodes extracted were adjusted to a final volume of 40ml water in a beaker.

**Nematode Assay for Visual Identification Method**

Nematodes extracted were identified to genus level whenever possible and counted under an inverted microscope (Fluovert, Leitz Wetzlar, Germany). Nematodes were categorized into five trophic groups: bacterivores, fungivores, herbivores, omnivores or predators (Yeates, 1993). Nematode richness was determined by counting the total number of taxa (mostly at the genus level with the exception of Rhabditidae). Additional nematode community indices were calculated included the Simpsons index of diversity (Simpson, 1949), maturity index (MI) (Bongers, 1998), enrichment index (EI), structure index (SI), and channel index (CI) (Ferris, 2001) were calculated.
**Statistical Analysis**

Data were subjected to one-way analysis of variance (ANOVA) using the general linear model (GLM) procedure in Statistical Analysis System (SAS Institute, Cary, NC). Total nematode number was log transformed \([\log_{10} (x +1)]\) prior to ANOVA to normalize the data. The percentage of predator and omnivore nematodes and diversity data were subjected to square root SQRT \(x\) prior to ANOVA to normalize the data. Untransformed arithmetic means of all data are presented. Data for all community indices were not transformed prior to analysis. All four sites were ranked for nematode indices and parameter. The qPCR results were used to calculate modified community indices based on the selected representative nematode guilds. Ecosystems were also ranked based on molecular-based nematode community indices. Ranking of ecosystems were compared between the molecular-based methods to the visual identification.

**Nematode Assay for qPCR Method**

Another 40 ml nematode extraction (from another 125 cm\(^3\) soil sub-sample) was processed for DNA extraction and real-time PCR (qPCR).
DNA Extraction from Environmental Sample

Nematode were collected from soil samples and picked into a sterilized 1.5ml eppendorf tube filled with 200μl distilled water. DNA from the samples were extracted using the PureLink™ DNA Extraction Kit (Invitrogen) with a protocol modified by adding bead-beating and heating steps as the protocol described previously in chapter 2.

Standard Curves for qPCR

To generate standard curves for the qPCR, the plasmid constructed with the universal qPCR primer amplicons from nematode guilds and known numbers of nematode genomic DNA were used. There were two different strategies while setting the standard curves for the five universal qPCR primer sets. One strategy utilized the plasmids constructed with each guild plasmid at 5 different dilutions (1x, 10x, 100x, 1000x, 10000x) plus one negative control. Six data points were converted to copies of plasmid in order to enrich the nematode sample DNA concentration calculation. QPCR data was then compared to the standard curve to obtain the corresponding plasmid copy number from which to determine guild member number within the environmental sample. The other strategy employed used DNA of a known number of nematodes. Nematode 18S rDNA was assumed to have 55copies in the
genome (Stricklin, 2005). However, the variation of copy number between species/genus can be considerable. The rDNA copy number varies between *C.elegans* and *C. briggsae* 2 fold (Stein, 2003). Therefore, to generate standard curves, genomic DNA extracted from a known number of nematodes of each guild (Ba2, F2-1, F2-2, Om4/Om5/P5 and P4) were serial diluted (1x, 10x, 100x, 1000x, 10000x). Each dilution data point represented exact nematode numbers, therefore by using the equation of standard curve, further nematode guild abundance could be achieved.

**qPCR Assay**

For the qPCR, amplifications were performed in real-time with 2µl 10X LightCycler® RNA Master SYBR Green I (Roche, USA), 1µl of 10 mM of F/R primer, 1.6µl of MgCl₂ and 2µl guild plasmid DNA (Ba2, F2-1, F2-2, Om4/ Om5/ P5 and P4, including 1X, 100X, 1000X, 10,000X and 100,000X serial dilution) in a final volume of 20µl. The reaction was performed in a 20µl carousel tube in a LightCycler® 1.5 Instrument (Roche, USA). The manufacturer recommended universal thermal protocol was used: 10 minutes preheating at 95°C for initial template denaturation and activation of hot start polymerase; this preparation was followed by 50 cycles of 95°C for 15 seconds, annealing temperature 58°C for 8 seconds, and extension temperature 72°C for 5 seconds followed by a 1 cycle melting curve analysis of 65
Following the suggestion of manufacture, each universal qPCR primer set had two replications plus one negative control within the same reaction. The results were collected and calculated to nematode copy number based on the estimated nematode 18S rDNA copy number (plasmid DNA standard curve) or genomic DNA standard curve.

To confirm the qPCR amplicon size, qPCR products were collected from the 20µl carousels by reversing the carousels containing qPCR product and placing them into a 1.5 ml eppendorf followed by centrifugation for 30 seconds at 8,000 × g, the qPCR products were then collected in the eppendorfs, carousels were discarded and qPCR products were analyzed by DNA electrophoresis.

Results

Nematode community analysis from visual identification-

Nematode parameters including richness, total number of nematodes, dominance, diversity, F/(F+B), and percentage of different feeding group of nematodes differed among sites. From visual identification, the richness (number of genera) was greatest in the pineapple field with nematode genera from bacterivores, fungivores, herbivores, omnivores and predators,
followed by forest site with nematode genera from bacterivores, fungivores, herbivores, omnivores and predators (Table 4.1). The organic and beach site were not significantly different (Table 4.2). The organic site consisted of 10 genera and the forest site consisted of 20 genera. The beach site had the richness of 14 genera while there were 29 genera found within the pineapple site
Table 4.1 List of nematode genera found in four soil ecosystems by visual identification.

<table>
<thead>
<tr>
<th>Soil ecosystem/site</th>
<th>Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic Site</td>
<td><em>Rhabditidae, Acrobelodies, Filenchus, Eucephalobus, Aphelenchodies, Aphelenchus, Prismatolaimus, Rotylenchulus reniformis</em> and Mononchus</td>
</tr>
<tr>
<td>Forest Site</td>
<td><em>Rhabditidae, Acrobelodies, Filenchus, Eucephalobus, Aphelenchodies, Aphelenchus, Prismatolaimus, Rotylenchulus reniformis, Meloidogyne, Helicotylenchus, Alaimus, Tylenchus, Mononchus, Pisilenchus, Tylencholaimellus, Mesodorylaimus, Laimydomus, Aporcellaimellus, Aporcellimus, and Neotylenchidae</em></td>
</tr>
<tr>
<td>Beach Site</td>
<td><em>Rhabditidae, Acrobelodies, Filenchus, Eucephalobus, Aphelenchodies, Aphelenchus, Prismatolaimus, Rotylenchulus reniformis, Pratylenchus, Mesocriconema, Tetracephalobus Aporcellimus, Aporcellimellus, Wilsonema and Mononchus</em></td>
</tr>
<tr>
<td>Pineapple Site</td>
<td><em>Rhabditidae, Acrobelodies, Filenchus, Cephalobus, Eucephalobus, Paracrebeles, Drileocephalobus, Pseudoacrobrlrd, Mohystera, Aphelenchodies, Aphelenchus, Tylenchus, Prismatolaimus, Rotylenchulus reniformis, Helicotylenchus, Paratylenchus, Mesocriconema, Meloidogyne, Rotylenchus, Alaimus, Tylenchus, Mononchus, Pisilenchus, Tylencholaimellus, Leptonchus, Mesodorylaimus, Aporcellaimellus, and Aporcellimus</em></td>
</tr>
</tbody>
</table>
The total number of nematodes in each site varied. The forest site and pineapple site had the highest total number of nematodes. The organic site and beach site showed low total number of nematodes (Table 4.2).

The dominance at the four sites did not show any significant differences from visual identification (Table 4.2). The diversity of organic, forest, beach and pineapple sites also did not differ (Table 4.2). The forest showed the highest fungivore to fungivore plus bacterivore F/(F+B) ratio. The organic site and pineapple site were similar and the beach site had the lowest fungivore to fungivore plus bacterivore F/(F+B) ratio (Table 4.2).

The total numbers of nematodes obtained by visual identification were different within these four sites (Table 4.2). The percentage of bacterivorous nematodes was highest at the beach site, followed by the organic and pineapple sites. The forest site showed the lowest percentage of bacterivore nematodes (Table 4.2). The percentage of fungivoreous nematodes differed among sites also. (Table 4.2) The organic site and forest site had the highest percentage of fungivore nematodes. The beach site and pineapple site showed the lowest percentage of fungivore nematodes (Table 4.2). The percentage of herbivore nematodes in the four sites was different. The forest site and pineapple site both showed a significantly high percentage of herbivore nematodes compared to the organic and beach site (Table 4.2). The
percentage of omnivore nematodes in all four sites was not different. The organic site did not contain any omnivore nematodes (Table 4.2). Lastly, the percentage of predatory nematodes was not different among the sites (Table 4.2).

The MI, EI, and SI calculated from the visual identification differed among the sites. Significant difference between forest site and beach site was found. No significant difference was seen between the organic and pineapple sites (Table 4.2). The enrichment index of organic, forest, beach and pineapple sites varied. The beach site was significantly different compare to other sites. The organic, forest and pineapple sites did not differ among themselves (Table 4.2). The structure index of four sites was not different statistically (Table 4.2). When the EI and SI were combined together, the four quadrants can represent disturbed/enriched, disturbed/depleted, stable/enriched and stable/depleted areas. The beach site fell into the stable/enriched quadrant, while the pineapple and forest sites were in disturbed/enriched quadrant. The organic site fell into disturbed/depleted quadrant (Fig 4.1).

The forest site statistically had the highest total number of nematodes with pineapple site, highest percentage of herbivorous nematodes, and highest percentage of fungivorous nematodes with organic site. The organic site had the lowest richness, total number of nematodes, and percentage of herbivorous nematodes, similar to the beach site. The EI and
EI/SI of organic site were also the lowest. Except to the highest percentage of fungivorous nematodes and EI, beach site have showed lowest richness, percentage of herbivorous nematodes, F/(F+B) and MI. Lastly, the pineapple site had the highest richness and total number of nematodes. However, the pineapple site had the lowest percentage of bacterivorous and fungivorous nematodes. The percentage of herbivore nematodes was highest in the pineapple site.

Overall, the beach site ranked first among nematode parameters/indices. The forest site followed and pineapple site was closely behind. A large separation was seen in the organic site and the pineapple site, the organic site ranked last in overall nematode community parameters/indices.
Table 4.2 Nematode parameters/indices and statistical analysis results from visual identification

<table>
<thead>
<tr>
<th>Nematode Parameters/Indices</th>
<th>Organic</th>
<th>Forest</th>
<th>Beach</th>
<th>Pineapple</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richness</td>
<td>6.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Number</td>
<td>153&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1235&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110&lt;sup&gt;b&lt;/sup&gt;</td>
<td>976&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diversity</td>
<td>1.9953&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3491&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8219&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3449&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F/(F+B)</td>
<td>0.525&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7925&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.125&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.435&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of Bacterivore</td>
<td>38.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.216&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of Fungivore</td>
<td>44.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.255&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of Herbivore</td>
<td>2.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.943&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of Omnivore</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.393&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.816&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of Predator</td>
<td>3.125&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.802&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.837&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.179&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maturity Index( MI)</td>
<td>1.995&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.213&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.575&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.963&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enrichment Index( EI)</td>
<td>26.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Structure Index( SI)</td>
<td>27.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Channel Index( CI)</td>
<td>92.308&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.029&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Means are an average of 4 replications. Means in a row followed by the same letter(s) do not differ according to Waller-Duncan k-ratio (k = 100) t-test based on arcsin (sqrt(x/100)) transformed, and non-transformed values for abnormally and normally distributed data, respectively.
Figure 4.1 Enrichment index/Structure index (EI/SI) of nematodes for the four sites by visual identification: an organic site in Waimanalo University of Hawaii Experimental Station, a pineapple field with *Crotalaria juncea* L. as a cover crop in Whitmore University of Hawaii Experiment Station, a forest site in University of Hawaii Lyon Arboretum, a beach site in Hanauma Bay Nature Park. All sites are on Oahu, HI.
qPCR Standard Curve

All standard curves were with acceptable error (<0.2), and also with a reasonable PCR efficiency between 1.8-2.2. The $R^2$ associated with these curves was between 0.9666-0.9996. The error of Ba2 standard curve was acceptable (<0.2) as 0.00989. The PCR efficiency was between 1.8-2.2 averaging 2.189. Moreover, the $R^2$ was 0.9966 (Fig 4.2.A). The standard curve developed by genomic DNA of known number Ba2 nematodes were shown, while 1,000x and 10,000x dilutions were not detected in the qPCR reaction. The $R^2$ of this curve was 0.9995 (Fig 4.2.B). The error of the F2-1 standard curve was acceptable (<0.2) as 0.0288, and PCR efficiency was between 1.8-2.2 averaging 2.013. Moreover, the $R^2$ was 0.9951 (Fig 4.2.C). Standard curve developed by genomic DNA of known number F2-1 nematodes had an $R^2$ of this curve 0.9887 (Fig 4.2.D). The error of the F2-2 standard curve was acceptable (<0.2) as 0.0263, and PCR efficiency was between 1.8-2.2. Moreover, the $R^2$ was an acceptable 0.9965 (Fig 4.2.E). Standard curve developed by genomic DNA of known number F2-2 nematodes only had 10x, 100x, and 1,000x serial dilutions and revealed a reasonable standard curve with an $R^2$ of 0.9668 (Fig 4.2.F). The error of the Om4/Om5/P5 standard curve was acceptable (<0.2) as 0.0000327, and PCR efficiency was between 1.8-2.2 as 1.806. Moreover, the $R^2$ was 0.9996 (Fig 4.3.A). Standard curve developed by genomic DNA of
known number Om4/Om5/P5 nematodes found no 10,000x signal in the qPCR assay. Therefore only 1x, 10x, 100x and 1,000x serial dilutions were used to generate a curve with an $R^2$ of 0.9973 (Fig 4.3.B). The error of the P4 standard curve was acceptable ($<0.2$) as 0.002, and PCR efficiency was between 1.8-2.2 as 1.897. Moreover, the $R^2$ was 0.9666 (Fig 4.3.C). Standard curve developed by gDNA of known number P4 nematodes were shown. While 1,000x and 10,000x serial dilution did not have any signal in the qPCR assay. Therefore only 1x, 10x, 100x serial dilution were shown. The $R^2$ of this curve was 0.9973 (Fig 4.3.D).
Figure 4.2 qPCR standard curves of Ba2, F2-1 and F2-2 nematode guilds

(A) The Ba2 standard curve constructed by serial dilution of Ba2 guild plasmid DNA. (B) The Ba2 standard curve constructed by serial dilution of Ba2 genomic DNA from 40 Ba2 nematodes. (C) The F2-1 standard curve constructed by serial dilution of F2-1 guild plasmid DNA. (D) The F2-1 standard curve constructed by serial dilution of F2-1 genomic DNA from 50 Ba2 nematodes. (E) The F2-2 standard curve constructed by serial dilution of F2-1 genomic DNA from 30 F2-2 nematodes. (F) The F2-2 standard curve constructed by serial dilution of F2-2 guild plasmid DNA. (A), (C) and (E) X axis was the log transformation of plasmid DNA concentration (g/ml). Y axis was the CP value calculated from the Roche Lightcycler software 4.05. (B), (D) and (F) X axis was the log transformation of nematode number. Y axis was the CP value calculated from the Roche Lightcycler software 4.05.
Figure 4.3 qPCR standard curves of Om4/Om5/P5 and P4 nematode guild

(A) The Om4/Om5/P5 standard curve constructed by serial dilution of Om4/Om5/P5 guild plasmid DNA. (B) The Om4/Om5/P5 standard curve constructed by serial dilution of Om4/Om5/P5 genomic DNA from 10 Om4/Om5/P5 nematodes. (C) The P4 standard curve constructed by serial dilution of P4 guild plasmid DNA. (D) The P4 standard curve constructed by serial dilution of P4 genomic DNA from 3 P4 nematodes. (A) and (C) X axis was the log transformation of plasmid DNA concentration (g/ml). Y axis was the CP value calculated from the Roche Lightcycler software 4.05. (B) and (D) X axis was the log transformation of nematode number. Y axis was the CP value calculated from the Roche Lightcycler software 4.05.
qPCR Assay

The qPCR result of each site was shown individually with the melting peaks of five guild qPCR, in order to ensure the specificity of primer sets in the qPCR assay (Fig 4.4-4.7). In addition, DNA electrophoresis of qPCR product was showed in figure 4.8-4.11 and results of qPCR values calculated to nematode copy number was organized in table 4.4-7.

For the forest site, the Ba2 and F2-1 qPCR primers were specific while only primer dimers were detected in the negative control (NTC) (Fig 4.4.A, B). Whereas the F2-2, Om4/Om5/P5 and P4 qPCR primer sets only showed signals from determined site samples but none from negative controls (Fig 4.4.C, D, E). For the organic site, the result of F2-1 qPCR primer had the specificity to the determined site samples, while only primer dimers were detected in the negative control (NTC) (Fig 4.5.B). Whereas Ba2, F2-2, and P4 qPCR primer sets only showed signals from determined site samples but none from negative controls in the organic site (Fig 4.5. A, C, E). Moreover, the Om4/Om5/P5 qPCR primer set was not able to detect any signal neither from organic site sample DNA or the negative control (Fig 4.5.D). For the beach site, Ba2, F2-1, F2-2, Om4/Om5/P5 and P4 qPCR primer sets showed signals but only primer dimers were observed from the negative controls (Fig 4.6.A-E). Compared to forest
and organic sites’ qPCR melting peaks, the negative controls of beach site samples showed more non-specific signals which proved to be primer dimers by DNA electrophoresis analysis. For the pineapple site, the Ba₂, F₂-1, F₂-2, Om₄/Om₅/P₅ and P₄ qPCR primer sets showed signals but only primer dimers from the negative controls (Fig 4.7.A-E). Similar to the beach site qPCR melting peaks, the negative controls of pineapple site samples showed more non-specific signals of primer dimers by DNA electrophoresis analysis.
Figure 4.4 Melting peaks of five qPCR primer sets from nematodes found in a forest site. (A) Ba2 qPCR primer sets. (B) F2-1 qPCR primer sets. (C) F2-2 qPCR primer sets. 1, 2 indicate sample replication. 3 indicates negative control.
Figure 4.4 (continued) Melting peaks of five qPCR primer sets from nematodes found in a forest site

1, 2 indicate sample replication. 3 indicates negative control.
Figure 4.5 Melting peaks of five qPCR primer sets from nematodes found in an organic site

(A) Ba2 qPCR primer sets, (B) F2-1 qPCR primer sets, (C) F2-2 qPCR primer sets. 1, 2 indicate sample replication; 3 indicates negative control.
Figure 4.5 (continued) Melting peaks of five qPCR primer sets from nematodes found in an organic site

(D) Om4/Om5/ P5 qPCR primer sets. (E) P4 qPCR primer sets. 1, 2 indicate sample replication. 3 indicates negative control.
Figure 4.6 Melting peaks of five qPCR primer sets from nematodes found in a beach site.

- (A) Ba2 qPCR primer sets.
- (B) F2-1 qPCR primer sets.
- (C) F2-2 qPCR primer sets.

1, 2 indicate sample replication.
3 indicates negative control.
Figure 4.6 (continued) Melting peaks of five qPCR primer sets from nematodes found in a beach site.

(D) Om4/Om5, P5 gPCR primer sets. (E) P4 gPCR primer sets. 1, 2 indicate the sample replication. 3 indicates negative control.
Figure 4.7 Melting peaks of five qPCR primer sets from nematodes found in a pineapple site. (A) Ba2 qPCR primer sets. (B) F2-1 qPCR primer sets. (C) F2-2 qPCR primer sets. 1, 2 indicate sample replication. 3 indicates negative control.
Figure 4.7 (continued) Melting peaks of five qPCR primer sets from nematodes found in a pineapple site. 1, 2 indicate sample replication, 3 indicates negative control. (D) Om4/Om5/ P4 primer sets. (E) P5 qPCR primer sets.
DNA electrophoresis of qPCR product-

The electrophoresis results of the forest site showed that Ba2-1 and -2 replications, F2 -1-1, -2, F2-2-1, -2, Om4/Om5/P5-1, -2 and P4-1, -2 replications have amplicon size as expected (Figure 4.8), while one of the P4 replicate had a primer dimer of a 60 bp (Figure 4.8 lane 10). The forest NTC (negative control) showed no observable qPCR amplicons.

For the organic site, Ba2-1 and -2 replications, F2 -1-1, -2, F2-2-1, -2 and P4-1, -2 replications have amplicon size as expected (Figure 4.9). No qPCR amplicon was amplified from the Om4/Om5/P5-1, -2 replications with Om4/Om5/P5 qPCR primer (Figure 4.9 lane 7-8). The NTC (negative control) of organic site qPCR product analyzed in DNA electrophoresis showed that none of the NTCs from five qPCR primer sets have observable qPCR amplicons.

For the beach site, non-specific signals were observed during melting curves from each qPCR primer set. Ba2-1 and -2 replications, F2 -1-1, -2, F2-2-1, -2, Om4/Om5/P5-1, -2 and P4-1, -2 replications have amplicon size as expected (Figure 4.10). The NTC (negative control) of beach site qPCR had no observable qPCR amplicons, but only primer dimers.

Lastly, pineapple site, similar melting peak figures as the beach site were observed with
non-specific signals from each qPCR primer set. Ba2-1 and -2 replications, F2-1-1, -2, F2-2-1, -2, Om4/Om5/P5-1, -2 and P4-1, -2 replications have amplicon size as expected (Figure 4.11).

The NTC (Negative control) of pineapple site qPCR product analyzed in DNA electrophoresis showed that none of the NTCs from five qPCR primer sets have observable qPCR amplicons, but primer dimers (Fig 4.11 lane 7-9, 12, 15).
Figure 4.8 Electrophoretic analysis of the qPCR products amplified from forest site DNA samples with guild qPCR primers in 3.5% agarose gels. Designed amplicons were Ba2(159 bp), F2-1 (153 bp), F2-2 (85 bp), Om4/Om5/P5 (139 bp), P4(196 bp).

- lane M: 50 bp-500 bp DNA markers (GeneMark, Taiwan)
- lane 1: Forest-Ba2-1 qPCR product
- lane 2: Forest-Ba2-2 qPCR product
- lane 3: Forest-F2-1-1 qPCR product
- lane 4: Forest-F2-1-2 qPCR product
- lane 5: Forest-F2-2-1 qPCR product
- lane 6: Forest-F2-2-2 qPCR product
- lane 7: Forest-Om4/Om5/P5-1 qPCR product
- lane 8: Forest-Om4/Om5/P5-2 qPCR product
- lane 9: Forest-P4-1 qPCR product
- lane 10: Forest-P4-2 qPCR product
- lane 11: Forest-Ba2 NTC qPCR product
- lane 12: Forest-F2-1 NTC qPCR product
- lane 13: Forest-F2-2 NTC qPCR product
- lane 14: Forest-Om4/Om5/P5 NTC qPCR product
- lane 15: Forest-P4 NTC qPCR product
Figure 4.9 Electrophoretic analysis of the qPCR products amplified from organic site DNA samples with guild qPCR primers in 3.5% agarose gels. Designed amplicons were- Ba2 (159bp), F2-1 (153 bp), F2-2 (85 bp), Om4/Om5/P5 (139 bp), P4(196 bp).

lane M: 50 bp-500 bp DNA markers (GeneMark, Taiwan)

lane 1: Organic-Ba2-1 qPCR product
lane 2: Organic-Ba2-2 qPCR product
lane 3: Organic-F2-1-1 qPCR product
lane 4: Organic-F2-1-2 qPCR product
lane 5: Organic-F2-2-1 qPCR product
lane 6: Organic-F2-2-2 qPCR product
lane 7: Organic-Om4/Om5/P5-1 qPCR product
lane 8: Organic-Om4/Om5/P5-2 qPCR product
lane 9: Organic- P4-1 qPCR product
lane 10: Organic- P4-2 qPCR product
lane 11: Organic-Ba2 NTC qPCR product
lane 12: Organic-F2-1 NTC qPCR product
lane 13: Organic-F2-2 NTC qPCR product
lane 14: Organic-Om4/Om5/P5 NTC qPCR product
lane 15: Forest-P4 NTC qPCR product
Figure 4.10 Electrophoretic analysis of the qPCR products amplified from beach site DNA samples with guild qPCR primers in 3.5% agarose gels. Designed amplicons were: Ba2(159bp), F2-1 (153 bp), F2-2 (85 bp), Om4/Om5/P5 (139 bp), P4(196 bp).

lane M: 50 bp-500 bp DNA markers (GeneMark, Taiwan)

lane 1: Beach-Ba2-1 qPCR product
lane 2: Beach-Ba2-2 qPCR product
lane 3: Beach-F2-1-1 qPCR product
lane 4: Beach-F2-1-2 qPCR product
lane 5: Beach-F2-2-1 qPCR product
lane 6: Beach-F2-2-2 qPCR product
lane 7: Beach-Om4/Om5/P5-1 qPCR product
lane 8: Beach-Om4/Om5/P5-2 qPCR product
lane 9: Beach- P4-1 qPCR product
lane 10: Beach- P4-2 qPCR product
lane 11: Beach-Ba2 NTC qPCR product
lane 12: Beach-F2-1 NTC qPCR product
lane 13: Beach-F2-2 NTC qPCR product
lane 14: Beach-Om4/Om5/P5 NTC qPCR product
lane 15: Beach-P4 NTC qPCR product
Figure 4.11 Electrophoretic analysis of the qPCR products amplified from pineapple site DNA samples with guild qPCR primers in 3.5% agarose gels. Designed amplicons were- Ba2(159 bp), F2-1 (153 bp), F2-2 (85 bp), Om4/Om5/P5 (139 bp), P4(196 bp).

lane M: 50 bp-500 bp DNA markers (GeneMark, Taiwan)
lane 1: Pineapple-Ba2-1 qPCR product
lane 2: Pineapple-Ba2-2 qPCR product
lane 3: Pineapple-F2-1-1 qPCR product
lane 4: Pineapple-F2-1-2 qPCR product
lane 5: Pineapple-F2-2-1 qPCR product
lane 6: Pineapple-F2-2-2 qPCR product
lane 7: Pineapple-Ba2 NTC qPCR product
lane 8: Pineapple-F2-1 NTC qPCR product
lane 9: Pineapple-F2-2 NTC qPCR product
lane 10: Pineapple-Om4/Om5/P5-1 qPCR product
lane 11: Pineapple-Om4/Om5/P5-2 qPCR product
lane 12: Pineapple-Om4/Om5/P5 NTC qPCR
lane 9: Pineapple- P4-1 qPCR product
lane 10: Pineapple- P4-2 qPCR product
lane 15: Pineapple-P4 NTC qPCR product
Result of qPCR CP values calculated to nematode copy number

The CP value (where the reaction’s fluorescence increase during log phase of amplification) and nematode 18S rDNA copy number (also estimated nematode number in genomic DNA standard curve) of four sites showed that nematode 18S rDNA copy number of Ba2, F2-1, F2-2 and Om4/Om5/P5 were very different between the plasmid DNA standard curve and the genomic DNA standard curve, while P4 data was similar (Table 4.3-4.6). Since plasmid DNA standard curve is relatively quantification of the nematode 18S rDNA without sufficient gene copy number information. Therefore these two standard curves could be compared by ranking of soil ecosystems but not the absolute value.

Overall, the nematode copy number derived from two different standard curves- plasmid DNA standard curve and gDNA standard curve were varied. The nematode 18S rDNA abundance of Ba2, F2-1 and F2-2 nematode guilds were very distinct within sites- forest, beach and pineapple. Furthermore, Om4/Om5/P5 nematode numbers were distinct in sites except the organic site where no Om4/Om5/P5 nematode was detected. The P4 nematode copy number was on a similar trend within all four sites.
Table 4.3 The CP value/nematode copy number of a forest site
qPCR CP values of forest site samples and calculated nematode copy number from plasmid DNA standard curve or genomic DNA standard curve

<table>
<thead>
<tr>
<th>Nematode Guild</th>
<th>CP value/calculated nematode copy number from plasmid DNA standard curve</th>
<th>CP value/calculated nematode copy number from genomic DNA standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-1</td>
<td>24.2/ 1148</td>
<td>24.2/ 67</td>
</tr>
<tr>
<td>Ba2-2</td>
<td>24.81/ 1066</td>
<td>24.81/ 62</td>
</tr>
<tr>
<td>F2-1-1</td>
<td>24.29/ 1134</td>
<td>24.29/ 4</td>
</tr>
<tr>
<td>F2-1-2</td>
<td>24.01/ 1395</td>
<td>24.01/ 5</td>
</tr>
<tr>
<td>F2-2-1</td>
<td>29.43/ 49</td>
<td>29.43/ 1</td>
</tr>
<tr>
<td>F2-2-2</td>
<td>29.64/ 41</td>
<td>29.64/ 1</td>
</tr>
<tr>
<td>Om4/Om5/P5-1</td>
<td>24.03/ 21787</td>
<td>24.03/ 66</td>
</tr>
<tr>
<td>Om4/Om5/P5-2</td>
<td>24.3/ 18690</td>
<td>24.3/ 54</td>
</tr>
<tr>
<td>P4-1</td>
<td>32.74/ 14</td>
<td>32.74/ 17</td>
</tr>
<tr>
<td>P4-2</td>
<td>32.91/ 12</td>
<td>32.91/ 15</td>
</tr>
</tbody>
</table>
Table 4.4 The CP value/nematode copy number of an organic Site
qPCR CP values of organic site samples and calculated nematode copy number from plasmid DNA standard curve or genomic DNA standard curve

<table>
<thead>
<tr>
<th>Nematode Guild</th>
<th>CP value/calculated nematode copy number from plasmid DNA standard curve</th>
<th>CP value/calculated nematode copy number from genomic DNA standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-1</td>
<td>21.16/ 10135</td>
<td>21.16/ 806</td>
</tr>
<tr>
<td>Ba2-2</td>
<td>21.19/ 9931</td>
<td>21.19/ 787</td>
</tr>
<tr>
<td>F2-1-1</td>
<td>21.03/ 10078</td>
<td>21.03/ 33</td>
</tr>
<tr>
<td>F2-1-2</td>
<td>21.01/ 10228</td>
<td>21.01/ 33</td>
</tr>
<tr>
<td>F2-2-1</td>
<td>23.83/ 49</td>
<td>23.83/ 19</td>
</tr>
<tr>
<td>F2-2-2</td>
<td>23.82/ 41</td>
<td>23.82/ 19</td>
</tr>
<tr>
<td>Om4/Om5/P5-1</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Om4/Om5/P5-2</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>P4-1</td>
<td>32.18/ 18</td>
<td>32.74/ 22</td>
</tr>
<tr>
<td>P4-2</td>
<td>32.85/ 10</td>
<td>32.91/ 16</td>
</tr>
</tbody>
</table>
Table 4.5 The CP value/nematode copy number of a beach site
qPCR CP values of beach site samples and calculated nematode copy number from plasmid DNA standard curve or genomic DNA standard curve

<table>
<thead>
<tr>
<th>Nematode Guild</th>
<th>CP value/calculated nematode copy number from plasmid DNA standard curve</th>
<th>CP value/calculated nematode copy number from genomic DNA standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-1</td>
<td>21.91/ 6771</td>
<td>21.91/ 505</td>
</tr>
<tr>
<td>Ba2-2</td>
<td>22.09/ 5993</td>
<td>22.09/ 440</td>
</tr>
<tr>
<td>F2-1-1</td>
<td>20.83/ 12980</td>
<td>20.83/ 8</td>
</tr>
<tr>
<td>F2-1-2</td>
<td>21.04/ 11115</td>
<td>21.04/ 7</td>
</tr>
<tr>
<td>F2-2-1</td>
<td>31.67/ 7</td>
<td>31.67/ 0.1</td>
</tr>
<tr>
<td>F2-2-2</td>
<td>32.01/ 6</td>
<td>32.01/ 0.1</td>
</tr>
<tr>
<td>Om4/Om5/P5-1</td>
<td>24.58/ 14171</td>
<td>24.58/ 39</td>
</tr>
<tr>
<td>Om4/Om5/P5-2</td>
<td>24.48/ 14999</td>
<td>24.48/ 42</td>
</tr>
<tr>
<td>P4-1</td>
<td>42.05/ 0.01</td>
<td>32.74/ 0.01</td>
</tr>
<tr>
<td>P4-2</td>
<td>40.39/ 0.02</td>
<td>32.91/ 0.03</td>
</tr>
</tbody>
</table>
Table 4.6 The CP value/nematode copy number of a pineapple site
qPCR CP values of pineapple site samples and calculated nematode copy number from plasmid DNA standard curve or genomic DNA standard curve

<table>
<thead>
<tr>
<th>Nematode Guild</th>
<th>CP value/calculated nematode copy number from plasmid DNA standard curve</th>
<th>CP value/calculated nematode copy number from genomic DNA standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba2-1</td>
<td>23.9/ 1976</td>
<td>23.9/ 124</td>
</tr>
<tr>
<td>Ba2-2</td>
<td>23.25/ 3070</td>
<td>23.25/ 204</td>
</tr>
<tr>
<td>F2-1-1</td>
<td>21.15/ 11529</td>
<td>21.15/ 37</td>
</tr>
<tr>
<td>F2-1-2</td>
<td>21.24/ 10788</td>
<td>21.24/ 35</td>
</tr>
<tr>
<td>F2-2-1</td>
<td>26.77/ 396</td>
<td>26.77/ 3</td>
</tr>
<tr>
<td>F2-2-2</td>
<td>26.78/ 393</td>
<td>26.78/ 3</td>
</tr>
<tr>
<td>Om4/Om5/P5-1</td>
<td>27.66/ 2773</td>
<td>27.66/ 4</td>
</tr>
<tr>
<td>Om4/Om5/P5-2</td>
<td>27.92/ 2392</td>
<td>27.92/ 4</td>
</tr>
<tr>
<td>P4-1</td>
<td>32.69/ 13</td>
<td>32.69/ 17</td>
</tr>
<tr>
<td>P4-2</td>
<td>32.96/ 10</td>
<td>32.96/ 15</td>
</tr>
</tbody>
</table>
Overall Ranking of Four Sites-

Not every index and parameter was calculated from qPCR due to insufficient data. Richness, percentage of herbivorous nematodes, Enrichment Index (EI), Channel Index (CI), diversity, and dominance were not calculated. Nematode indices/parameters that were calculated are compared with the visual identification data (Table 4.7). The table indicated that the ranking calculated from two standard curves, including percentage of omnivore and predator, Maturity Index (MI) and Structure Index (SI) were the same. For the overall comparison between the rankings of the four sites, the visual identification revealed the beach site as the best ecosystem among those four sites followed by the forest, pineapple and organic sites. The qPCR method ranked the forest site followed by the beach, organic and pineapple sites (plasmid DNA standard curve). The ranking result derived from the genomic DNA standard curve showed the forest site as the best, followed by the beach and pineapple site and last is the organic site. The qPCR overall ranking from genomic DNA standard curve showed trends similar to the visual identification while plasmid DNA standard curve gave a distinct result. Furthermore, nematode parameters of genomic DNA standard curve result and visual identification including percentage of bacterivores, fungivores, omnivores and SI were similar, whereas the percentage of omnivore and SI were similar between plasmid standard
curve result and visual identification. The overall result of nematode community analysis has been conducted by both traditional and molecular methods. Besides the comparison of nematode community analysis obtained by both methods (Table 4.7), another table showing the comparison of the nematode parameters/indices that each method can obtain was generated (Table 4.8). With this research, molecular tools were able to obtain the total number of nematodes, F/(F+B), percentage of bacterivore, fungivore, predator and omnivore nematodes, also the SI and EI.
Table 4.7 Overall Ranking of four sites- including organic site, forest site, beach site and pineapple site. Each site had three ranking number of each nematode parameter or indices, V indicates visual identification, G indicates the genomic DNA standard curve and P indicates the plasmid DNA standard curve. Ranking was given from 1 to 4, 1 indicates the highest calculation result and 4 indicates the lowest calculation result.

<table>
<thead>
<tr>
<th>Nematode Parameter s/Indices</th>
<th>Total Number</th>
<th>F/(F+B)</th>
<th>% of Bacterivores</th>
<th>% of Fungivores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V  G  P</td>
<td>V  G  P</td>
<td>V  G  P</td>
<td>V  G  P</td>
</tr>
<tr>
<td>Organic</td>
<td>3  1  2</td>
<td>2  3  4</td>
<td>2  1  1</td>
<td>1  1  2</td>
</tr>
<tr>
<td>Forest</td>
<td>1  4  4</td>
<td>1  2  3</td>
<td>4  4  4</td>
<td>2  3  4</td>
</tr>
<tr>
<td>Beach</td>
<td>4  2  1</td>
<td>4  4  2</td>
<td>1  2  3</td>
<td>4  4  3</td>
</tr>
<tr>
<td>Pineapple</td>
<td>2  3  3</td>
<td>3  1  1</td>
<td>3  3  2</td>
<td>3  2  1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nematode Parameter s/Indices</th>
<th>% of Omnivores</th>
<th>% of Predators</th>
<th>MI</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V  G  P</td>
<td>V  G  P</td>
<td>V  G  P</td>
<td>V  G  P</td>
</tr>
<tr>
<td>Organic</td>
<td>4  4  4</td>
<td>2  3  3</td>
<td>2  4  4</td>
<td>4  4  4</td>
</tr>
<tr>
<td>Forest</td>
<td>2  1  1</td>
<td>3  1  1</td>
<td>1  1  1</td>
<td>2  1  1</td>
</tr>
<tr>
<td>Beach</td>
<td>1  2  2</td>
<td>1  4  4</td>
<td>4  3  2</td>
<td>1  2  2</td>
</tr>
<tr>
<td>Pineapple</td>
<td>3  3  3</td>
<td>4  2  2</td>
<td>3  2  3</td>
<td>3  3  3</td>
</tr>
</tbody>
</table>

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Table 4.8 Comparison of the nematode parameters/indices achieved by traditional identification and molecular identification. Traditional identification indicates visual identification, while molecular identification indicates qPCR method. The nematode parameters/indices were selected for this research.

<table>
<thead>
<tr>
<th>Nematode Parameters/Indices</th>
<th>Traditional Identification</th>
<th>Molecular Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richness</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Total Number</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>% of Bacterivores</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>% of Fungivores</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>% of Herbivores</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>% of Predators</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>% of Omnivores</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>F(F+B)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dominance</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Diversity</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Maturity Index (MI)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Enrichment Index (EI)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Structure Index (SI)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Channel Index (CI)</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
Discussion

The comparison of the traditional visual identification to a molecular qPCR approach to the nematode community analysis did result in a similar conclusions for the four sites. The nematode community analysis conducted by the visual identification method revealed that overall the beach site showed the most enriched/stabled environment with a low MI. This was due to its high percentage of bacterviores and fungivores. The beach site was located at Hanauma Bay, Oahu, which has been a tourist site for years. However the samples were collected far from regular activities. The staff does not disturb the soil yet provide the Scaevola taccada plants with fertilization. The beach site nematode community analysis indicated a typical soil ecosystem (Bongers, 1998; Bongers, 1999; Neher, 2001). The pineapple and forest sites showed similar trends on EI/SI ratio, being enriched but disturbed. The percentage of herbivore nematodes within these two sites were statistically similar, however, the pineapple soil ecosystem was categorized as a depleted/disturbed environment due mainly to the presence of the herbivores Rotylenchulus reniformis (Barker, 1994; Schmitt, 2000; Sipes, 2005) which was not found in other sites. The pineapple site had a Crotalaria juncea cover crop, which explains the high EI or enriched environment (Wang, 2002, 2002, 2003). The pineapple site was tillaged and had been fumigated 1.5 year
previously and the SI indicated a disturbed environment which reflected the disturbed/enriched environment as in Figure 4.1. Finally, even with cover crop intercropping, the percentage of herbivores was still higher due to the *R. reniformis* population. The forest site has been categorized as a natural ecosystem, and the EI accurately indicated an enriched environment. Nevertheless, the SI of the forest site showed disturbance. The samples from the forest site were collected at Lyon Arboretum, which has many different plant species in a forest. The location where samples were actually collected had a nursery with many young seedlings nearby that was maintained by the staff of the Arboretum. The disturbed environment maybe related to the activity in the nursery. Moreover, the Arboretum does not regularly control weeds which supports *Helicotylenchus* (Wouts, 1933) therefore resulting in the large number of *Helicotylenchus* been identified only in this site. Therefore, the visual and molecular nematode community analysis of the pineapple and forest site was reflective of the actual situation.

From visual identification results, the organic site, not only showed a depleted/disturbed environment, but the richness and total number of nematodes were low. The fungivore nematodes population was high resulting in an unbalanced environment of bacterial/ fungal decomposition. Organic soil ecosystems are usually categorized as enriched (organic
amendments) and disturbed (without chemical pesticide/insecticide, manual tillage is used more often). However, our visual and molecular nematode community analysis showed a depleted environment undergoing little bacterial/fungal decomposition. The depleted condition might be due to teaching purposes of this site. The area was not used for more than 9 months; therefore the nutrients were depleted as shown by the parameters derived from the visual identification. There were no omnivore nematodes found in the organic site and few predatory nematodes were observed. This disturbed environment might be due to the frequent tillage activities during the teaching semester. Moreover, the depleted condition of soil ecosystem with few nutrient sources could also contribute to the decrease of omnivore and predatory nematodes (Yeates, 1993; Bongers, 1999). Both the visual identification method has proved the assumption of four soil ecosystems in the research were distinctly different.

The qPCR method did not allow the calculation of all nematode parameters/indices. Richness, diversity, dominance, number/percentage of herbivore, enrichment index (EI) and channel index (CI) could not be calculated from the qPCR assay. The hypothesis of this research was to achieve most nematode parameters/indices while richness and diversity, dominance and diversity required whole scale sequencing or DGGE assay (De Ley, 2005;
Griffiths, 2006; Okada, 2008; Wang, 2008). The number/percentage of herbivore nematodes could not be calculated because neither; the number of herbivore nematode genera across different guilds and the variation in their 18S rDNA. The amount of primer sets required would be problematic in a qPCR methodology. EI and CI were not calculated because the Ba1 nematode copy number is needed and we were unable to develop a Ba1 qPCR primer set (Chapter 2). This might be overcome by developing a qPCR primer set for Ba1/Ba2 and applied to qPCR assay together with Ba2 qPCR primer.

The visual identification result might have revealed the actual condition of the four soil ecosystems. Although the results have shown that the two methods did not reveal a similar trend on all aspects. However, four nematode parameters/indices out of eight, which are percentage of bacterivores, fungivores, omnivores and SI, displayed similar trends for gDNA standard curve result, while percentage of omnivore and SI were similar between plasmid DNA standard curve and visual identification. This research was valuable for the development of one molecular method to answer nematode community analysis. Strategies to design nematode guild specific primer sets were developed. Moreover, the specificity of the primers were challenged either with database alignment, PCR method or DNA sequencing and successfully proved the efficiency of these nematode guild primer sets. During the
nematode community analysis employing visual identification, the process was advised under trained experts, therefore the nematode community analysis result for the four sites were reliable. The qPCR method was designed to use SYBR Green Dye as the fluorescence. SYBR green I fluorescent dye has the virtue of being easy to use because (i) it has no sequence specificity, (ii) it can be used to detect any PCR product, and (iii) it binds to any double-stranded DNA (Rasmussen, 1998). However, this virtue has a drawback, as the dye binds also to any non-specific product including primer-dimers, a melting curve analysis could be employed to overcome this disadvantage. From the melting curve of the qPCR method, it has showed very specific amplification, and primer-dimers were made. Therefore the results of qPCR were reliable as well. Nematode community analysis utilizing qPCR method was completed by obtaining the nematode 18S rDNA copy number from two types of standard curves- plasmid DNA and genomic DNA. The results between those two standard curves showed distinct nematode 18S rDNA copy number (estimated nematode number for gDNA standard curve) in nematode guild Ba2, F2-1, F2-2 (all four sites) and Om4/ Om5 and P5 (except organic site) while P4 showed a similar trend. However, when the nematodes’ copy number was applied to nematode parameters/indices, including percentage of bacterviores/fungivores/omnivores/predators and Enrichment index (EI) the calculations did not change. The trends were consistent even with distinct nematode copy number.
The ranking result of individual nematode parameters or indices reveal similar trends among visual identification/ qPCR with the plasmid DNA standard curve with SI and percentage of Omnivore while four out of eight nematode parameters or indices (% of bacterivores, fungivores, omnivores and SI) were similar between qPCR with gDNA standard curve and visual identification. The plasmid DNA and gDNA standard curve had same ranking for percentage of omnivore, predator, MI and SI. Several possible reasons exist for this result. The partial total number was used since the visual identification included the number of herbivorous nematodes which were not detected in the qPCR assay. Therefore the ranking result for total number of nematodes was very different. Also, principally, plasmid DNA standard curves were used as the absolute quantification of qPCR method under the condition of knowing the copy number of detecting gene in target organisms (Madania, 2005; Holeva, 2006; Ward, 2006; Watzinger, 2006; Nakhla, 2010). Nevertheless, calculating the nematode number from the plasmid DNA standard curve was based on the assumption that all nematodes have the same copy number of 18S rDNA. The copy number of certain genes (i.e. 18S rDNA and 5S rDNA) in Nematoda has not been studied other than in C. elegans (Sulston, 1974; Ellis, 1986; Consortium, 1998; Stricklin, 2005). The 18S rDNA gene used here was determined to have ~55copies in C. elegans, but no information on copy number is available for other nematodes. Many studies have addressed the correlation/variation
between rDNA copy number and genome size in eukaryotes. Also, *C. briggsae* has shown gene copy number variation with *C. elegans*. Gene copy number might be different even within a genus (Nelson, 1989; Prokopowich, 2003). The plasmid DNA standard curve could show relative abundance of nematodes and the gDNA standard curve was employed and might provide a more accurate nematode copy number. Furthermore, the plasmid DNA and gDNA both showed similar trend to percentage of omnivore and SI, which were the nematode indices measuring the nematodes that higher weighted and rare. Which might suggest that less overestimation was with these nematodes by using qPCR assay.

The gDNA standard curve was conducted to overcome the difficulties of unknown 18S rDNA copy number, the DNA of known number of guild nematodes were extracted and serial diluted to a reasonable standard curve, i.e. 40 Ba2 nematodes; 50 F2-1 nematodes; 30 F2-2 nematodes; 10 Om4/ Om5/ P5 nematodes and 3 P4 nematodes. However, the DNA recovery during the extraction process might have resulted in the nematode copy number underestimated from the gDNA standard curve (Donn, 2008). Usually the problem of DNA recovery can be adjusted by quantifying the gDNA and dividing by the genome size of the organism. Nevertheless, the genome size of Nematoda is unknown except for several species in genus *Caenorhabditis*. Thus, the gDNA standard curve might not be as accurate as
expected.

The qPCR primer sets developed in this research are capable to use in soil ecosystems outside Hawaii for a relative comparison between soil ecosystems since the trend of the soil ecosystems ranking from qPCR assay was reliable with relative nematode abundance. Further study to answer the question of nematode gene copy number can be achieved by sequencing the nematodes’ Chromosome I, which is the location of the ribosomal DNA. Moreover, the number of tandem repeats actually results to the copy number of 18S ribosomal DNA (Sulston, 1974; Stricklin, 2005). The research can start with the sequencing of tandem repeat regions, and gradually extend to the whole chromosome. In this way, the copy number of 18S rDNA can be more accurately determined, and the qPCR method using 18S rDNA can be applied more widely (Hernault, 1988; Coghlan, 2005). The qPCR primer sets developed in this research have their specificity and affordability already and will be more extensively applied to nematode community analysis when estimating nematode copy number is more accurate.

The purpose of nematode community analysis is to contribute the assessment of soil health. The soil condition of ecosystem can be partially evaluated by both traditional visual identification and qPCR assay (gDNA standard curve). With more information of Nematoda
18S rDNA copy number, the qPCR primer sets and experiment design developed in this research will contribute to the soil health assessment from a different perspective.
Literature Cited


