Root-Knot Nematodes and Coffee in Nicaragua: Management Systems, Species Identification and Genetic Diversity

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Cover: Coffee plantation in Nicaragua, flowers, beans and seeds of coffee, coffee roots affected by *Meloidogyne exigua* and female of *M. exigua*.
(photos: Therese Bengtsson, Anna Zborowska, Arnulfo Monzón)

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Abstract

The coffee production is affected by several limiting factors such as parasitic nematodes. Plant-parasitic nematodes have a substantial economic impact on coffee production in most countries and they are regarded as one of the major limiting factors in coffee production and worldwide coffee losses have been estimated to about 15%.

The aim of this thesis is to contribute to the knowledge of the plant-parasitic nematodes associated with coffee production in Nicaragua, by studying the effect of coffee management, identification of *Meloidogyne* species and determination of the level of genetic diversity in the root-knot nematodes and coffee. This thesis includes results of the effect of coffee management systems on the occurrence of plant-parasitic nematodes, identification of root-knot nematodes using morphological characteristics, SCAR markers and DNA sequences; ISSR based genetic diversity of *M. exigua* and SSR based genetic diversity of *Coffea arabica* in Nicaragua.

Both *Meloidogyne* and *Pratylenchus* species were found in both conventional and organic management systems. A higher population of *Meloidogyne* spp. occurred under conventional management with no shade and with shade from nitrogen fixer species while higher populations of *Pratylenchus* spp. occurred in both conventional and organic management, shaded with non-fixer nitrogen species. Analyses of root-knot nematode isolates from Nicaraguan coffee plantations based on perineal patterns, SCAR markers and DNA sequence revealed the presence of *Meloidogyne exigua* and *M. incognita*, of which the former has a wider distribution in the country. DNA sequences analysis revealed that this species has unique variable sites within the 18S and 28S rDNA region, which are not present in the rest of the *Meloidogyne* species including in those sharing similar reproductive mechanisms. These variable sites are useful for developing new species specific SCAR markers that can be used in the diagnosis of *M. exigua*. The assessment of the genetic diversity of *M. exigua* populations based on ISSR markers revealed a high genetic diversity in this species. Similarly, the genetic diversity analyses of *C. arabica* populations using SSR markers revealed a significant variation among different varieties and the highest diversity was obtained in variety Catimor,

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Dedication

To my beloved children: Oscar Miguel and Gisselle Cristina

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numeral in the text:

I   Herrera, I., Bryngelsson, T. & Monzón, A. (2011). Occurrence of *Meloidogyne* spp. and *Pratylenchus* spp. in conventional and organic coffee management systems. Nematropica 41: 80-88.

II Herrera, I., Bryngelsson, T., Monzón, A. & Geleta, M. (2011). Identification of coffee root-knot nematodes based on perineal pattern, SCAR markers and nuclear ribosomal DNA sequences. Nematologia Mediterranea, Vol. 39: *in press*.

III Herrera, I., Bryngelsson, T., Monzón, A. & Geleta, M. Inter simple sequence repeats (ISSR) based analysis of the genetic diversity of *Meloidogyne exigua* from coffee plantations in Nicaragua. (Submitted Manuscript).

IV Herrera, I., Bryngelsson, T., Monzón, A. & Geleta, M. Genetic diversity of arabica coffee (*Coffea arabica* L.) in Nicaragua as estimated by microsatellite markers. (Manuscript).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Isabel Cristina Herrera Sirias to the papers included in this thesis was as follows:

I   Set up and carried out all experimental work, collected soil samples, extracted and identified soil nematodes in the lab, analyzed data and wrote the manuscript in collaboration with co-authors.

II Collected coffee root samples in Nicaraguan coffee plantations, planted coffee and tomato seeds and inoculated plants with *Meloidogyne* spp. in greenhouse, extracted DNA from females of *Meloidogyne* spp., planned and carried out all laboratory work, analyzed data and wrote manuscript together with the supervisors.

III Planned and carried out all laboratory work, extracted DNA from females of *Meloidogyne* species, analyzed data and wrote manuscript together with the supervisors.

IV Collected coffee beans in Nicaraguan coffee plantations, processed the coffee beans, planted the seeds in a greenhouse, planned and carried out all laboratory work, extracted DNA, analyzed data and wrote manuscript together with the supervisors.

1   Introduction

Coffee is an important crop in tropical and subtropical countries. It is regarded as a major source for foreign exchange, and contributes significantly to the economy of developing countries (Waller *et al*., 2007). The main regions of coffee production over the world are South America (45%), Asia and Oceania (26. %), Africa (13 %) and Mexico and Central America (16%) (ICO, 2010).

The Nicaraguan coffee production amounts to 10 % of the total production of Mexico and the Central American countries (ICO, 2010), and represents consistently about 25% of the export income for the country (Pirotte *et al*., 2006). Environmentally, coffee is one of the most important crops in the country where about 95% of the produced coffee is grown under shady conditions serving as refuge for many animal species, such as bird and insects (MIFIC, 2005; Perfecto *et al*., 1996). The coffee produced in Nicaragua is classified as "washed arabigo" and the main varieties cultivated are: Caturra, Bourbon, Catuaí and Typica (MAGFOR, 2006). Coffee is produced in high (600 to 1500 m a.s.l) and low altitude zones (below 600 m a.s.l.) (Guharay *et al*., 2000). Coffee cultivation varies from traditional to intensive management systems. In the traditional system, low density of coffee trees, high density of shade trees and low use of chemicals are common, in contrast to the intense system where coffee trees are grown at high densities under sunny conditions and a high use of chemicals (Perfecto *et al*., 1996; Sánchez-de León *et al*., 2006).

Several plant-parasitic nematodes species are associated with coffee cultivation and they cause great losses to coffee-growers and to the local economy (Campos & Villain, 2005). The major species affecting coffee are *Meloidogyne* spp. and *Pratylenchus* spp. (Villain *et al*., 2000; Barbosa *et al*., 2004). Damage by these nematodes have been reported throughout the world including Latin America (Campos & Villain,2005; Anzueto, 1993; Villain *et al*., 2002) and Central America (Salas & Echandi, 1961; Schieber & Soza,

1960; Fernández, 1968), where nematodes of these two genera are often found parasitizing the coffee trees simultaneously (Villain *et al*., 2002). Root-knot nematode (*Meloidogyne* spp.) represents the major threat in all major coffee-growing (*Coffea arabica* L.) areas throughout the world. *M. exigua and M. incognita* are regarded as the most damaging species on coffee in Latin America (Carneiro & Cofcewicz, 2008). Root-lesion nematodes (*Pratylenchus* spp.) are also very common and cause serious damage to coffee. Eight species of *Pratylenchus* have been found on coffee, *P. coffeae*, being the most widely distributed in coffee plantations (Campos & Villain, 2005).

Plant-parasitic nematodes have a substantial economic impact on coffee in most coffee-producing countries. They are regarded as the major limiting factor in coffee production and worldwide coffee losses have been estimated to approximately 15% (Campos & Villain, 2005). Once root-knot nematodes are established in a coffee plantation, they build up over time and certain conditions such as type and content of organic matter, can increase the nematode population (Waller *et al*., 2007). In addition, the majority of coffee varieties cultivated in Central American countries are susceptible to root-knot and root-lesion nematodes (Bertrand *et al*., 2000). Chemical control, although the most reliable means to control root-knot nematodes, is expensive, with a minimal effectiveness when the symptoms of nematodes are already observed. These chemicals are also toxic to humans and environment (Villain *et al*, 2008) and therefore new strategies to control plant-parasitic nematodes are needed, e.g. integrated management practices and tolerant coffee varieties (Bertrand *et al*., 2000).

In Central America the first signs of damage caused by *Meloidogyne* and *Pratylenchus* species were reported in 1937. All countries except Honduras have widespread infestation by nematodes in most of coffee-growing regions (Villain *et al*., 1999; Campos & Villain, 2005). Several studies have been carried out to characterize *Meloidogyne* species using various morphological criteria but they are unpractical and insufficient. Studies of esterase-izoenzymes have revealed a great diversity among *Meloidogyne* species (Hernández, *et al*., 2004). Some *Meloidogyne* species attack coffee and *M. exigua, M. incognita, M. coffeicola* and *M. paranaensis* are regarded as the most common and damaging species (Carneiro & Cofcewicz, 2008), *M. exigua* is probably the most widespread species in coffee plantations in Central America (Villain *et al*., 2007). *Pratylenchus* species in Central American coffee plantations have been studied by morphological characterization, pathogenicity tests and molecular tools. These studies have revealed a distinct

genetic diversity and high a degree of pathogenicity in field experiments and commercial plantations (Anzueto, 1993; Hervé, 1997).

In Nicaragua, the presence of plant-parasitic nematodes in coffee plantations has been reported previously (Sequeira, 1977; Rosales-Mercado & Reyes-García, 1991; Herrera & Marbán-Mendoza, 1998), although the species were not identified. The species report on root-knot nematodes (RKN) from Nicaraguan coffee plantations was by Hernández *et al.* (2004) and Herrera *et al.* (2011; Paper II) who reported *M. exigua* and *M. incognita* in the South Pacific and North Central region.

No detailed research has been done earlier on plant parasitic nematodes in coffee plantation in Nicaragua. Therefore, studies on the occurrence of plant-parasitic nematodes in conventionally and organically managed coffee plantation in the country were conducted in this thesis. In addition, identification and investigations on the genetic diversity of coffee RKN and a study of the genetic diversity of coffee varieties in Nicaraguan coffee plantation was done.

We hope that the results of the present study will provide a basic knowledge about plant-parasitic nematode in Nicaraguan coffee plantations and that these insights will be useful in the design of programs for integrated management of parasitic nematodes and in the breeding of resistant or tolerant coffee varieties for a sustainable coffee production in Nicaragua.

2   Background

2.1   Coffee as a crop – General information

Coffea arabica L. is native to the southwest highlands of Ethiopia and it is the most widespread cultivated coffee species around the world (Cubry *et al.*, 2008). The arabica coffee cultivation started in Yemen five centuries ago. Around 1700 it was spread to South Asia and in the early 18th century, progenies of a single plant from Indonesia spread via Amsterdam and Paris to Latin America (Vieira, 2008). Coffee is now extensively cultivated in about 60 tropical and subtropical countries. Twenty-five countries produce over one million 60 kg bags/year (Waller *et al.*, 2007); the top coffee producing countries being Brazil, Colombia, Indonesia, Vietnam, Mexico, Ethiopia, India, Guatemala, Cote D Ivoire and Uganda (Faostat, 2010). The production of arabica coffee is predominant in America, except for Brazil which also produces robusta coffee. In Africa, Vietnam and Indonesia robusta coffee is predominant (Ha & Shively, 2008; Vieiria, 2008).

The genus *Coffea* belongs to the family Rubiaceae, which consists of approximately 500 genera and over 6000 species (Waller *et al.*, 2007). Although the genus *Coffea* consists of 103 species (Cubry *et al.,* 2008), only two species are commercially exploited: *C. arabica* and *C. canephora*. *C. arabica* is autogamous, although about 10% of cross pollination can occur (Krug & Carvalho, 1951) and it is the only allotetratploid species (2n = 4x = 44) in the genus; the rest of the species are diploid (2n = 22) (Vieiria, 2008). *C. arabica* is adapted to high altitudes while *C. canephora* has its origin in the forests of tropical Africa (Charrier & Berthaud, 1985). High quality coffee is associated with *C. arabica*, preferred by consumers due to its aromatic characteristics, low bitterness and low content of caffeine (Wellman, 1961; Lécollier *et al.*, 2009). *C. canephora*, is characterized by strong bitterness and a high content of caffeine (Lécolier *et al.*, 2009).

C. arabica has been cultivated for about 200 years in Central America. Although it has a good quality it is susceptible to serious pests and diseases, e.g. the coffee berry borer (*Hypothenemus hampei*) and coffee leaf rust (*Hemileia vastatrix*) and plant-parasitic nematodes, particularly *Meloidogyne* species and *Pratylenchus* especies (Staver *et al*., 2001). Two cultivars of arabica coffee: Typica and Bourbon are widely distributed, which have high yielding and good quality of beverage but limited by a narrow genetic base (Fernández *et al*., 2001; Anthony *et al*. 2002)

2.2 History of coffee and its cultivation in Central America and Nicaragua

Central America is the second most productive coffee region in the world after Brazil and contributes to approximately 15% of the world production, despite the small geographic area (Villain *et al*., 2008). In this region, coffee production is an important economic activity for all Central American countries where more than 700,000 ha are used for this crop. Coffee is predominantly grown by smallholders and the 25% of rural population are engaged in this activity (Perfecto *et al*., 1996; MIFIC, 2005). Coffee is produced under different conditions ranging from traditional systems to modern monoculture (Perfecto *et al*., 1996).

Central America is a region with optimal ecological conditions for coffee production, although its potential is not fully exploited and their productivity still remains low (MIFIC, 2005; Perfecto *et al*., 1996; Villain *et al*., 2008). Coffee crop is mainly grown in highland areas, usually at 800 to 1,600 m asl, characterized by heavy rainfall (from May to October) followed by a dry period (November to April). Coffee is grown as a monoculture since the end of 19th century, without crop rotation which favours the development of nematode population and their dissemination (Villain *et al*., 2008).

Coffee has traditionally been cultivated with shade trees but from the 1970s many coffee growers changed to cultivation under full sun combined with highly productive cultivars and external input of chemicals. The new management created problems with soil erosion, weeds and nematodes which are less problematic when shady conditions were used (Staver *et al*., 2001; Villain *et al*., 2008). Today, more sustainable production systems are receiving interest from the growers. The use of shade trees is considered beneficial as the shade decrease exposure of coffee plants to extreme heat or cold, reduces wind speed, controls erosion, and helps to maintain soil fertility (Beer *et al.,* 1998; Somarriba *et al.,* 2004). The organic debris produced by the shade trees favour

the development of a macrofauna and microflora antagonistic to plant-parasitic nematodes (Stirling, 1991).

Coffee was introduced to the Carazo Province in Nicaragua in 1820 and gradually spread to the rest of the country (Kuhl, 2004). Approximately 130,000 ha are dedicated to the coffee cultivation. The North Central region represents 79% of the national coffee production. Coffee contributes to about 25% of the export of Nicaragua exports income and is one of the main sources of employment in the rural areas and accounts for 13% for the country as a whole (MIFIC, 2005). Considering that about 96% of the area of coffee cultivation is under shady conditions, coffee is an environmentally very important crop, serving as a refuge for birds and other animals and insects (MIFIC, 2005). The management of coffee production in Nicaragua varies widely from open sun monocultures to multistrata coffee polycultures with different levels of external input (Somarriba *et al*., 2004). Within shaded coffee cultivation, organic production has opened up a new market for farmers and better chances to increase their income (Beer *et al*., 1998). The organic production system is based on diversity, recycling and biological processes and has low environmental impact and it is important in terms of environmental services such as water and soil conservation and carbon sequestration (Figueroa-Zevallos *et al*., 1996). The fall of international coffee prices has opened up good opportunities to organic coffee compared to coffee produced under conventional management (Beer *et al*., 1998; Somarriba *et al*., 2004).

There are many limiting factors affecting coffee production. During the last three decades coffee pests have increased significantly due to the intense coffee production based on the use of synthetic pesticides and the promotion of coffee varieties potentially productive under sunny conditions (Staver *et al*., 2001). Severe damage from root-knot nematodes and lesion-root nematodes have been reported in Nicaragua coffee plantations.

2.3   Plant-parasitic nematodes - General information

Nematodes are the most numerous metazoans on earth. They are free-living or parasites of plants or animals and are present in almost all possible habitats. They are aquatic organisms and their movement depends on moisture (Decraemer & Hunt, 2006). Nematodes are attracted to the plant roots by factors which are still not fully understood (Decraemer & Hunt, 2006). Plant-parasitic nematodes exhibit three types of parasitism, ectoparasitic, semi-endoparasitic and endoparasitic. The endoparasites nematodes induce sophisticated trophic systems of nurse cells or sincytia in their host; the female

becomes obese lose mobility (Hunt &Manazanilla-López, 2005). Plants with the root system affected by nematodes show symptoms as stunting, chlorosis, wilting and reduced yield. The symptoms in the aerial part of the plant are due to the poor ability of the root system to deliver water and nutrients. These symptoms are generally confused with water or nutrient deficiencies (Hunt & Manazanilla-López, 2005). Problems with plant-parasitic nematodes are present in all areas where crops are grown; the most obvious damage occurs in tropical or sub-tropical regions due to the high temperature, long growing seasons and the large number of susceptible crops which will gradually increase the nematode populations year by year (Mai, 1985). Plant-parasitic nematodes are responsible for global agricultural losses close to $80 billion annually (Agrios, 2005).

2.3.1 *Meloidogyne* spp.

Among the many genera of plant-parasitic nematodes of economic importance, *Meloidogyne* species represent the most widely distributed and damaging group responsible for the majority of losses (Sasser & Freckman, 1987).

Eighty *Meloidogyne* species has been described until now (Karssen & van Hoenselaar, 1998), which display different modes of reproduction. Few species of *Meloidogyne* are amphimictic and produce cross-fertilized eggs e.g. *M. carolinensis, M. megatyla*, and *M. pini* (Jepson, 1987). Most RKN reproduce trough parthenogenesis showing variations. Some species such as *M. chitwoodi*, *M. exigua* and *M. fallax* reproduce by cross fertilization and by meiotic parthenogenesis and when males are present they also reproduce by cross fertilization. Others *Meloidogyne* species reproduce by obligatory mitotic parthenogenesis. These species *e.g. M. arenaria, M. incognita* and *M. incognita* are considered as the most important species due to a worldwide distribution and a wide range of hosts (Triantaphyllou, 1966; Van der Beek *et al*., 1998). *Meloidogyne* species are obligate and sedentary endoparasites of the roots, tubers and corms and have evolved very specialized and complicated relationships with their host. Typically they reproduce and feed in a specialized cell within the plant root, inducing small or big galls or root-knots, producing malfunctioning root systems. The damage may include various levels of stunting, poor of vigour and wilting under moisture stress (Hussey, 1985; Moens *et al*., 2009).

2.4   Plant-parasitic nematodes on coffee in Central America

Several species of plant-parasitic nematodes cause yield losses and have a significant impact on the economy of coffee producing countries (Campos &

Villain, 2005). Seventeen species of *Meloidogyne* have been reported to attack coffee and four of these, *M. exigua*, *M. incognita*, *M. coffeicola* and *M. paranaensis* are regarded as the most common and damaging species (Carneiro & Cofcewicz, 2008). In Central America, six *Meloidogyne* species have been reported in coffee. *M. exigua* is the most widely spread species and has been reported from Costa Rica, Nicaragua and Honduras (Garcia *et al*., 2009; Muniz *et al*., 2009; Barbosa *et al*., 2010). *M. arenaria* has been reported from El Salvador, *M. arabicida* from Costa Rica and *M. hapla* from Guatemala (Hernández *et al*., 2004). A study based on esterase isozymes showed the presence of *M. incognita* in Costa Rica, Guatemala and El Salvador (Villain *et al*., 2007). *M. mayaguensis* has been reported from coffee plantations on Cuba (Rodríguez *et al*., 1995; Hernández *et al*., 2001).

M. exigua and *M. incognita* are probably the most damaging species on coffee in Latin America. Yield losses caused by these nematodes have been estimated up to 10% in Costa Rica (Bertrand *et al*., 1997) and 45% in Brazil (Barbosa *et al*., 2004). The wide range of *Meloidogyne* species reported from coffee is related to their large diversity in pathogenicity (Hernández, 1997; Villain *et al*., 1999) and research is going on to identify and characterize these species in order to design a reliable pest management strategy and coffee breeding programs.

2.5   Systematics of root-knot nematodes (RKN) and methods of identification

Accurate identification of plant-parasitic nematodes is needed for more precise research and effective control (Powers, 1992). Identification of plant-parasitic nematodes is however, not an easy task especially in the case of certain taxa or when more than one species occur in the same sample (Abrantes *et al*., 2004).

Meloidogyne species show wide morphological variations among and within species, making their identification difficult. Some *Meloidogyne* species can be identified by their host-specificity, such as *M. carolinensis*, which only has two host species (Einsenback, 1982; Hartman & Sasser, 1985) but the identification can not only be based on host specificity (Einsenback, 1982). Others factors that can make identification difficult is a poor description of nematode species or high similarity between well-known species such as *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Hartman & Sasser, 1985). To improve the identification of RKN a rapid, reliable and easy technique is needed.

2.5.1 Traditional methods

The identification of *Meloidogyne* species is an extraordinary task, even for well qualified taxonomists. Traditional diagnosis of nematodes is based on detailed measurements and comparisons of morphological structures (Carneiro & Cofcewicz, 2008). Morphological differences between species are often based on the average of measurements from a population of individuals, which can be a problem when the population is a mixture of related species (Powers, 2004). The perineal pattern is the most common taxonomic feature used to identify most RKN (Campos & Villain, 2005). However, identification based only on the perineal pattern of egg-lying females can be uncertain, since some species, e.g. *M. paranaensis*, *M. konaensis*, *M. izalcoensis* and *M. mayaguensis* may show a perineal pattern similar to *M. incognita*, which may lead to misidentification of the species. The identification of *M. incognita* recently reported from coffee in Guatemala and El Salvador, might be uncertain as recent classification using enzyme phenotyping detected *M. paranaensis* and *M. izalcoensis* instead of *M. incognita* (Carneiro & Cofcewicz, 2008). When species identification is solely based on perineal patterns, the identification process should be done carefully, using only properly collected, prepared and mounted mature females (Carneiro & Cofcewicz, 2008). In addition, morphological characteristics of juveniles and mature males can be helpful in the identification of *Meloidogyne* species (Jepson, 1983a; Jepson, 1983b). Although morphological and morphometric data can be very useful they might be inconclusive as they often vary considerably within a population and it may not be sufficient to distinguish closely related *Meloidogyne* species (Hirschmann, 1986; Zijlstra, 2000; Carneiro & Cofcewicz, 2008). Cytological, cytogenetic and molecular information need to be used as a complement to morphological data in the identification process, particularly for *Meloidogyne* species (Moens *et al*., 2009).

2.5.2 Biochemical Methods

Analysis of proteins by polyacrylamide gel electrophoresis is an important method for species differentiation of RKN. Some enzymes, especially esterases, malate dehydrogenase and alpha-glycerolphosphate dehydrogenase exhibit distinct patterns for each species. The most used biochemical method for identification of RKN is the esterase pattern (Moens *et al*., 2009). The first use of isozyme phenotypes on the identification of RKN was done by Esbenshade & Triantaphyllou (1985). In a survey involving 300 populations from 65 countries, they reported esterase pattern for 16 *Meloidogyne* species (Esbenshade & Triantaphyllou, 1990). Carneiro *et al*. (2000) studied 111 populations of *Meloidogyne* from South America and found 18 esterase

phenotypes. Zu *et al*. (2004) found five esterase phenotypes from 46 populations of *Meloidogyne* species from China. Hernández *et al.* (2004) studied 29 isolates of *Meloidogyne* species from coffee plantations in four Central American countries and one isolate from Brazil and found six new multi-enzyme phenotypes. Isozyme phenotypes are considered an attractive system because of their relative stability within *Meloidogyne* species (De Waele & Elsen, 2007); nevertheless, some complications can occur due to intraspecific variants and difficulty in resolving size variants between species (*e.g. M. incognita* and *M. hapla*) and more than one enzyme is needed to confirm the species. In *M. exigua* poor signal intensity suggests the use of more than one female (Carneiro *et al*., 2000).

2.5.3 DNA -based methods

Molecular diagnosis of plant-parasitic nematodes has revolutionized our knowledge on the taxonomy and phylogeny of nematodes (Blok, 2005). There are several reasons why molecular data are more reliable for phylogenetic studies than morphological characters. DNA sequences are strictly heritable and the interpretation of molecular data is easier than morphological features. In addition, molecular characters are more abundant and are generated in a relatively shorter period of time as compared to morphological characteristics (Subbotin & Moens, 2006). The molecular diagnosis of plant-parasitic nematodes can be performed using bulk samples of females and second-stage juveniles (J2) or single J2 (Blok, 2005). The techniques are usually very sensitive and can detect specific species in mixed populations (Zijlstra, 2000; Subbotin *et al*., 2001). Some methods have been developed for molecular diagnosis directly from infected roots, galls or infected soil (Blok & Powers, 2009). The polymerase chain reaction (PCR) is the key tool in the study of plant-parasitic nematodes, and can be used for phylogenetic studies, molecular breeding for resistance and molecular diagnostics (Blok, 2005).

A range of DNA-based techniques is available for taxonomic or diagnostic purposes (Gonçalves de Oliveira *et al*., 2011). Ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) are among the most commonly DNA regions used for taxonomic and diagnostic purposes (Abrantes *et al*., 2004). The rDNA repeat unit, containing 18S, 28S and 5.8S coding genes and the internal transcribed spacer (ITS), external transcribed spacer (ETS) and intergenic spacer (IGS) have been widely used for phylogenetic and diagnostic studies (Hajibabaei *et al*., 2007; Blok, 2005). These regions have been very important for characterization of 15 species of plant-parasitic nematodes that are economically important and regulated in quarantine legislation (Blok, 2004). The 18S and 28S rDNA genes have been widely used for phylogenetic

purposes (Blok, 2005). For example, the 18S rDNA of 19 populations of *Meloidogyne* species was amplified and sequenced and three main clades were identified including the most important and disseminated species, *e.g. M. incognita, M. javanica* and *M. arenaria* (Tigano *et al.*, 2005). Similarly, the phylogeny of 12 species of *Meloidogyne* species was deduced using the 18S rDNA sequences, indicating that this region is useful in addressing phylogeny within *Meloidogyne* species (De Ley *et al.*, 2002). Holterman *et al.* (2009) constructed a phylogeny of 116 Tylenchida taxa based on full sequence of 18S rDNA. Landa *et al.* (2008) characterized three *M. hispanica* isolates from three different origins (Brazil, Portugal and Spain) using sequences from 18S, 5.8S ITS2 and D2-D3 of 28S and found identical sequences in all three isolates. *M. hispanica* from *M. incognita* and *M. arenaria* are difficult to distinguish by morphological and biological features. The analysis of the three rDNA regions demonstrated and supported the differentiation of *M. hispanica* from *M. incognita, M. javanica* and *M. arenaria.*

The first characterization of plant-parasitic nematodes was done using mitochondrial DNA, which is an interesting target for molecular diagnostics due to the high copy numbers of mtDNA in each cell and their fast rate of sequence evolution (Blouin, 2002). mtDNA is an excellent source for genetic markers for population genetics and species identification (Hu & Gasser, 2006). The mitochondrial DNA region has been used for identification of *M. mayaguensis* and to monitor the presence and distribution of this species. This region has been useful in distinguishing *M. mayaguensis* from *M. incognita* and *M. arenaria* (Blok *et al.* 2002).

A number of different kinds of molecular markers have been developed. The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification, and is used to find and to asses genetic diversity (Vos *et al.*, 1995). Qualitative and quantitative analysis have been carried out to evaluate genetic variation on populations of cyst nematodes and root-knot nematodes (Xue *et al.*, 1993). Fargette *et al.* (2005) studied the genetic diversity of *M. chitwoodi* and *M. fallax*, two major agricultural pests and found that *M. chitwoodi* display higher diversity than *M. fallax*. AFLP have proved to be useful to analyze the inter- and intra- specific genetic diversity in several organisms (Han *et al.*, 2000; Tooley *et al.*, 2002).

Random Amplification of Polymorphic DNA (RAPD) has been used to discriminate to interspecific and intraspecific relationships in *Globodera* and *Meloidogyne* species (Castagnone-Sereno *et al.*, 1994; Blok *et al.*, 1997b). It has been used to distinguish *M. incognita*, *M. javanica* and *M. arenaria* (Cenis, 1993). RADP markers that are different among different species can be

developed into sequence characterized amplified region (SCAR) markers that can be effectively used for the diagnosis of important pests such as *Meloidogyne* species (Blok & Powers, 2009). Their specificity and sensitivity varies and depends on the number of species and isolates evaluated. Several sets of SCAR primers can be used at the same time in multiple reactions to identify many species (Zijlstra, 2000; Randig *et al*., 2002). Zijlstra (2000) reported that SCAR markers specific to *M. chitwoodi*, *M. fallax* and *M. hapla* were amplified using DNA from eggs, juveniles and females. Tigano *et al*. (2010) detected 16 *M. enterolobii* isolates of different geographical origin using species-specific markers.

Inter simple sequence repeat (ISSR) a PCR-based molecular marker technique has proved to be highly useful for various applications including genetic diversity and population genetic structure studies of various species (Zietkiewicz *et al*., 1994; Geleta & Bryngelsson, 2009). ISSRs uses single primer for amplification of the target regions, which is a DNA segment present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction (Zietkiewicz *et al*., 1994). Carneiro *et al*. (2008) studied the diversity of *M. arenaria* using RAPD and ISSR markers and found high level of polymorphism among the isolates of this species.

Microsatellites or simple sequence repeats (SSRs) have been widely used for population genetics. Analysis of the variation in microsatellites in populations can give information about the population genetic structure and differences, genetic drift and the last common ancestor (Subbotin & Moens, 2006). SSR markers have been widely used for genetic diversity and populations studies of many plant species. SSR are highly polymorphic and widespread in plant genomes (Morgante & Olivieri, 1993). Ruas *et al*. (2003) used SSR markers to assess the genetic diversity and relationships among coffee trees (Maluf *et al*., 2005) and for evaluating coffee varieties with a narrow genetic base (Bredemeijer *et al*., 2002). Cubry *et al*. (2008) studied the genetic diversity of cultivated and wild coffee species (*Coffea arabica* L. and *C. canephora* Pierre ex Froehn.) used microsatellites and found a large amount of diversity in wild species. They also reported high heritability of microsatellite markers within *Coffea*, which can be useful for breeding applications. Baruah *et al*. (2003) studied nine microsatellite markers that revealed high polymorphism in 17 related coffee species suggesting their potential use as genetic markers for evaluating diversity and linkage analysis in coffee. In addition, molecular markers have been identified which are linked to a gene conferring resistance to *Colletotrichum kahawae* and *M. exigua*. This is

a promising option for control of diseases and pests by resistance breeding (Noir *et al*., 2003; Gichuru *et al*., 2008).

3   Objectives of the study

The aim of this thesis was to contribute to the knowledge of the plant-parasitic nematodes associated to coffee in Nicaragua, through studying the effect of coffee management, identification of *Meloidogyne* species and determination of the level of genetic diversity in the nematode and coffee populations within the country.

Specific objectives were to:

1. Compare the effect of different coffee management systems and shade trees on the population of *Meloidogyne* and *Pratylenchus* species in Nicaragua.

2. Identify and characterize root-knot nematodes in Nicaraguan coffee plantations using morphological markers, SCAR markers and nuclear ribosomal DNA sequences.

3. Investigate species variation and the genetic diversity in 18 isolates of *M. exigua* from coffee plantations in Nicaragua using ISSR markers.

4. Determine the level of genetic diversity and population genetic structure among the main varieties of *Coffea arabica* used in Nicaragua.

4 Materials and methods

4.1 Field experiment

The study was conducted from 2006 to 2008 in experimental coffee fields established in Masatepe, province of Masaya, located in the South Pacific coffee growing zone of Nicaragua. Based on climate and altitude, this zone is regarded as a dry and low elevation zone. The management systems evaluated were conventional with shade from nitrogen fixer species (*Inga laurina* and *Samanea saman*), conventional with shade from non-nitrogen fixer species (*Tabebuia rosea* and *Simarouba glauca*), conventional with no shade, organic with shade from nitrogen fixer species and organic with shade from non-nitrogen fixer species. The experimental field layout and descriptions of the treatments are presented in **paper I**.

4.2 Soil collection and nematodes extraction methods

Soil samples were collected in June 2006 and February 2007, June 2007 and February 2008, during the rainy (June) and dry (February) seasons. Five composite samples were randomly taken per plot. The extraction method used was sieves and cotton filter (75 μm pore size) and the number of nematodes was recorded by direct counting with a dissecting microscope. The numbers of *Pratylenchus* spp. and the second-stage juveniles (J2) of *Meloidogyne* spp. were recorded. Identification of *Meloidogyne* and *Pratylenchus* species at the genus level was based on morphological characteristics (**paper I)**.

4.3 Coffee root sample collection

Eighteen *Meloidogyne* isolates were collected from two coffee growing regions in Nicaragua. Fifteen isolates were from the South Pacific region and three isolates from the North Central region. All isolates were collected from coffee

roots and maintained on coffee (*Coffea arabica* L.) and tomato plants (*Solanum lycopersicum* L.) in a greenhouse with a mean temperature of 27 ºC (**paper II, III**).

4.4   Coffee berry collection

Twenty-six populations corresponding to eight *Coffea arabica* varieties were sampled. Ripe berries were collected directly from the coffee plantations during the harvest period of the coffee production cycle 2009-2010. The coffee seeds were planted in pots in a greenhouse with a mean temperature of 28ºC and plants were grown during approximately 70 days until the plants had the first true leaves (**paper IV**).

4.5   Perineal pattern of root-knot nematodes

For morphological identification, the perineal pattern of ten egg-lying females from each isolate was identified according to Taylor & Netscher (1974) and Hartman & Sasser (1985). When a high level of variation was observed, up to twenty perineal patterns were analyzed (**paper II**).

4.6   DNA extraction from nematodes and coffee (*Coffea arabica* L.)

DNA was extracted both from single and bulked females. Single female DNA was extracted from ten females using Extract-N-Amp™ Tissue PCR Kit (SIGMA) following the instructions provided by the supplier. For bulk DNA, forty females were used and DNA was extracted using a protocol originally developed for insects (Reineke *et al*., 1998) with minor modifications (**paper II, III**). For the study of the genetic diversity of coffee varieties, DNA was extracted from young and fresh leaves using a modified CTAB procedure as describe by Aga *et al*. (2003) (**paper IV**). The quality of DNA was analyzed by agarose gel electrophoresis and DNA concentration was determined using a Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden).

4.7   Polymerase chain reaction, DNA sequencing and fragment analysis

The 18S nuclear rDNA gene fragment was amplified using the primer-pair *MelF* and *MelR,* and primer-pair *D3A* and *D3B* were used for the 28S nuclear rDNA (Chen *et al*., 2003; Tigano *et al*., 2005). The conditions for the amplification reactions for each rDNA gene are described in **paper II.** Partial

sequences of the 18S and 28S rDNA were amplified and sequenced using DNA extracted from single females. The PCR products of the 18S and 28S rDNA were purified using a QIA quick PCR purification kit (QIAGEN GmbH, Germany) as recommended by the manufacturer. Five nanogram of the purified PCR product was added to a 1.5 ml Eppendorf tube for each sequencing reaction and the samples were dried overnight. Fifteen µl of Millipore water was added and the samples were sent to EUROFINS MWG Operon (Ebersberg, Germany) for sequencing with an ABI PRISM-3100 genetic analyzer (Applied Biosystems) (**paper II**).

4.8   Sequence characterized amplified regions (SCAR markers)

For SCAR marker analysis, three primer-pairs specific to *M. exigua*, *M. incognita* and *M. paranaensis* were used (Randig *et al.*, 2002). The PCR amplification was done using DNA from both single and bulked female nematodes. DNA was amplified using a Gene AMP PCR system 9700 thermocycler (**paper II**).

4.9   Inter-simple sequence repeats (ISSR)

Twelve primers that revealed polymorphism were selected for this study. PCR amplification was done using DNA from bulked females and the DNA amplification reactions were performed according to **paper III**. Separate PCR products were visualized using a DNA silver staining kit (GE Healthcare Bio-Sciences AB, Sweden) using a Hoefer Automated Gel Stainer (Pharmacia Biotech, USA) as recommended by the kit supplier.

4.10 Simple sequence repeats (SSR)

Twenty five SSR primer-pairs from different sources were initially screened and amplified PCR products were separated on readymade high resolution polyacrylamide gels (ETC Electrophorase-technik, Germany) and the gels were stained using a DNA silver staining kit (GE Healthcare Bio-Sciences AB, Sweden) as described in Geleta & Bryngelsson (2009). Twelve primer-pairs were selected for final analysis. The selected forward primers were fluorescently 5'-labeled with either 6FAMTM, VIC®, NEDTM or PET® florescent dyes. The reverse primers were PIG-tailed with "*GCTTCT*" to avoid non-template addition of a single nucleotide by *Taq* DNA polymerase to the PCR product as described in Ballard *et al.* (2002). The PCR products were multiplexed into two panels, each containing PCR products amplified by six

primer-pairs. Each multiplex panel included combinations of loci whose alleles would be separated by at least 80 bp from each other when labeled with the same florescent dye to avoid overlapping. The multiplexed PCR products were analyzed using an ABI Prism 3730 DNA Analyzer (Applied Biosystems).

4.11 Data analysis

Field experiment data were analyzed by a two-way ANOVA test using a split plot approach (PROC GLM) to compare the population level between treatments and between sample dates. Means were compared using the least significant difference (LSD) when statistical differences between treatments or between dates were detected. Partial sequences of the 18S and 28S rDNA were edited using BIODIT version 7.0.5 (Hall, 2005) and the sequences were visually inspected using SEQUENCE SCANNER version 1.0 (Applied Biosystems). Sequences were aligned using CLUSTAL X version 1.81 (Thompson *et al*., 1997) followed by manual adjustment. Maximum parsimony based phylogenetic analysis was carried out using PAUP* 4.0 Beta 10 (Swofford, 2000) based on the aligned sequences from the present study and from GenBank.

The ISSR band profiles were treated as dominant markers and data were scored as 1 for presence and 0 for absence of a DNA band at each locus across the 18 populations. In the case of SSR, each peak was considered as an allele at a co-dominant locus. Analysis of percentage of polymorphic loci and analysis of molecular variance (AMOVA) was conducted using POPGENE ver. 1.31 (Yeh & Boyle, 1979) and Arlequin ver. 2 (Schneider *et al*., 2000), respectively. The NTSYSpc program (Rohlf, 2000) was used to calculate Nei´s standard genetic similarity (Nei, 1972), cophenetic correlation coefficient, cluster analysis and bootstrapping, Jaccard's similarity coefficient and for principal coordinate analysis (PCoA). The Free Tree-Freeware program (Pavlicek *et al*., 1999) was used to generate Nei's standard genetic distance and for cluster analysis and bootstrapping. TreeView (Win32) 1.6.6 program (Page, 1996) was used to view the trees.

5 Summary of results and discussion

5.1 Occurrence of *Meloidogyne* and *Pratylenchus* species in different coffee management systems (Paper I).

Meloidogyne and *Pratylenchus* species were found in both conventional and organic coffee management systems. Both genera were found to attack coffee roots simultaneously, *Meloidogyne* being the most common. Predominance of *Meloidogyne* species (*e.g. M. exigua* and *M. paranaensis*) over *P. coffea* has also been reported from coffee plantations in Guatemala (Hervé *et al*., 2005). Our result suggests competition between individuals from the two genera. Several studies have earlier described competition between *Meloidogyne* and *Pratylenchus* with the predominance of *Meloidogyne* (Umesh *et al*., 1994; Chapman & Turner, 1975).

Meloidogyne populations were predominant in conventional management systems with no shade (open sun) compared to organic management. In contrast, *Pratylenchus* spp. showed low populations. One possible factor influencing the high density of *Meloidogyne* spp. in open sun in conventional management is the temperature requirement of these species, which is favoured by high temperatures while *Pratylenchus* spp. seem to be adapted to a wider range of temperature fluctuations, but with less capacity to compete. The high population density of *Meloidogyne* spp. in conventional management suggests that their occurrence is rather related to the input level than type of shade. The population density of *Pratylenchus* spp. was similar in both conventional and organic management systems and the occurrence of this genus could be related more to type of shade than input levels (**Table 1**). There is not much information that supports our results on the occurrence of plant-parasitic nematodes under different coffee management systems. Staver *et al*. (2001) mentioned that coffee production in conventional systems with high yielding varieties in open sun creates favourable conditions for numerous pests. Villain *et al*. (1999) reported that reducing shade in coffee plantations results in a reduction of organic matter, which is linked to increased nematode damage.

Carcache (2002) mentions that populations of *Meloidogyne* and *Pratylenchus* in coffee plantations in Costa Rica were higher in conventional compared to organic management.

Table 1. Effect of coffee management systems on the occurrence of *Meloidogyne* spp. and *Pratylenchus* spp. during 2006-2008. Masatepe, Masaya.

Coffee management	Shade type	Nematodes/200 g of soil			
		Meloidogyne spp.		*Pratylenchus* spp.	
		Mean (n=60)	LSD (α=0.05)	Mean (n=60)	LSD (α=0.05)
Conventional	*Tabebuia rosea*[2] and *Simarouba glauca*[1]	3470	ab	161	a
	Inga laurina[1,3] and *Samanea saman*[2,3]	4216	a	87	b
	No shade	3936	a	54	b
Organic	*Inga laurina*[1,3] and *Samanea saman*[2,3]	2824	ab	70	b
	Tabebuia rosea[2] and *Simarouba glauca*[1]	2337	b	157	a

[1] Evergreen; [2] Deciduos, [3] Nitrogen fixer

We found that the effect of shade tree species was different between the two genera, while the population of *Meloidogyne* spp. increased by shade from *Inga laurina* and *Samanea saman* (nitrogen fixer) in conventional systems. *Pratylenchus* spp. showed similar densities in both conventional and organic systems shaded by *Tabebuia rosea* and *Simarouba glauca* (non nitrogen fixer). Organic management systems with non nitrogen fixer shade species had the lowest densities of *Meloidogyne* spp. According to our results it seems that the shade trees have significant effect on the populations of *Meloidogyne* spp. and *Pratylenchus* spp. According to Araya (1994) the maintenance of high levels of soil organic matter by shade trees might help to stabilize the *Meloidogyne* and *Pratylenchus* populations below critical levels. Cruz *et al.* (1998) reported a high diversity of nematodes including predator nematodes from shaded coffee compared to open sun under conventional management. However, shade species could have an opposite effect, *e.g. Inga* spp. has been identified as an alternative host for coffee nematodes (Zamora & Soto, 1976; Aragón *et al.*, 1978).

The density of population of *Meloidogyne* spp. was higher in February than in June while no difference was observed for *Pratylenchus* spp. between sampling dates. The high population density of *Meloidogyne* in February can be due to the soil moisture present from the previous rainy season, which could have an effect on root growth dynamics in the coffee plantations (Inomoto & Oliveira, 2008). Similar results were found by (Cruz e*t al.*, 1998) in the same

coffee growing zone of Nicaragua. These results indicate that *Meloidogyne* spp. increase their population as long as the rainy season progress, but the mechanism behind this phenomenon is still unknown (Mc Sorley, 1997).

In summary, this study demonstrates that *Meloidogyne* and *Pratylenchus* species are significantly affected by the farming system. The different response of these species to different management systems and shade tree species should be considered when designing programs of integrated pest management for coffee.

5.2   Identification of coffee root-knot nematodes based on perineal pattern, SCAR markers and nuclear ribosomal DNA sequence (Paper II).

Eighteen isolates collected from coffee plantations across Nicaragua were identified based on perineal pattern, SCAR markers and partial sequences of 18S and 28S nuclear rDNA. The perineal patterns were variable but detailed observations of each isolate indicate the presence of *Meloidogyne exigua* in all isolates. In addition to *M. exigua, M. incognita* was also found in isolate M-15 from El Danubio. The analysis of a large number of egg-lying females from each isolate as suggested by Carneiro & Cofcewicz (2008) and following the method described by Taylor & Netscher (1974) and Hartman and Sasser (1985) resulted in two grouping of the nematodes into two distinct types, corresponding to *M. exigua* and *M. incognita*. According to our observations the *M. incognita* perineal pattern showed more variation than *M. exigua* (**Fig. 1**).
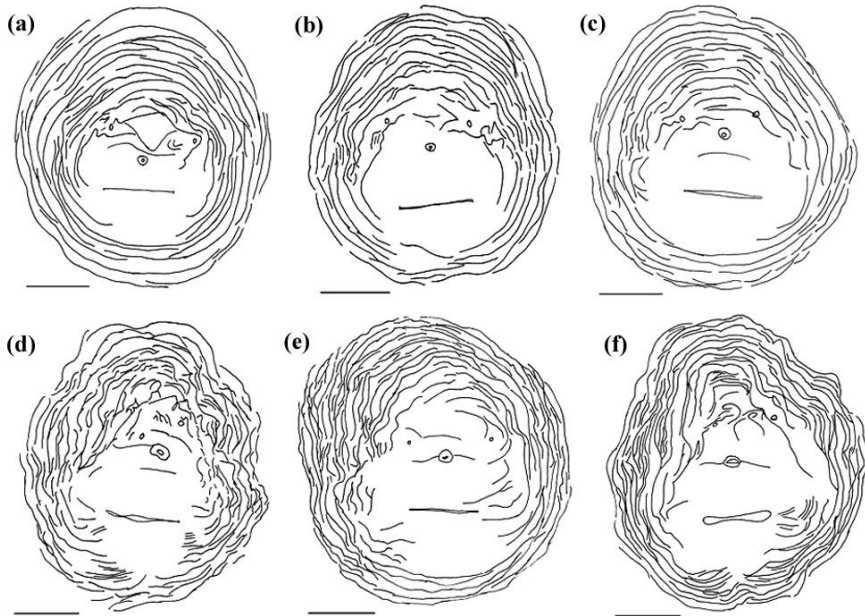
Figure 1. Variability of perineal patterns of *Meloidogyne exigua* (a, b, c) and *Meloidogyne incognita* (d, e, f) isolates identified from Nicaraguan coffee plantations. Scale bars: 20 μm.

Amplification of DNA from bulk and single females using the SCAR primers for *M. exigua*, resulted in a specific band of expected size, 562 bp. in all eighteen isolates. The *M. incognita* primers yielded a fragment of 399 bp in isolate M-15. No amplifications were obtained with the *M. paranaensis* primer-pair. The results were in complete agreement with the identification based on the perineal patterns. The application of SCAR marker analysis was a rapid and efficient tool in the identification of *Meloidogyne* species and their application on specimens that represent the perineal pattern variants identified the specimens either as *M. exigua* or *M. incognita*, suggesting the absence of other RKN species in the eighteen coffee plantations sampled (**Fig. 2**.)
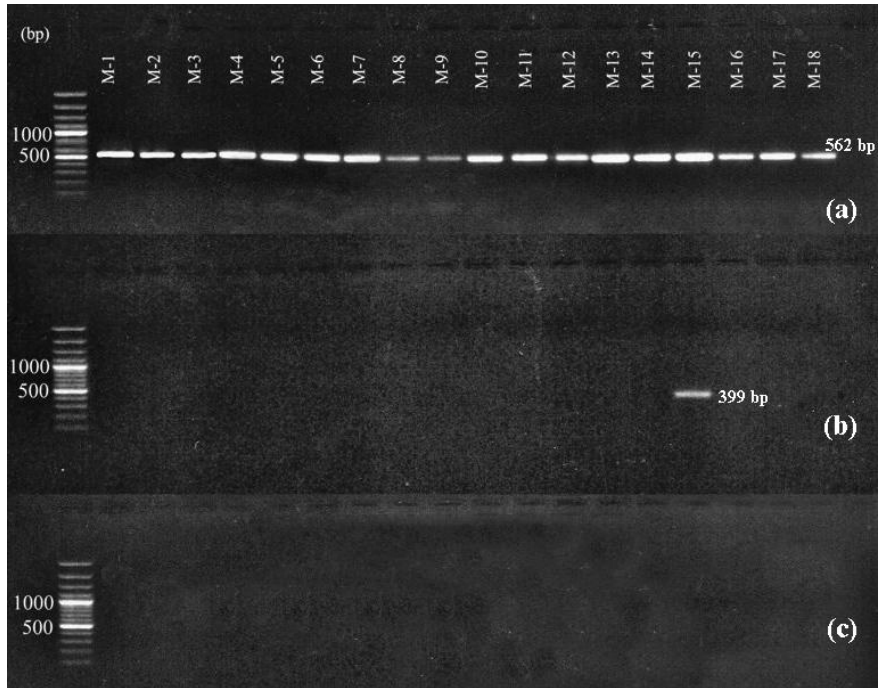
Figure 2. Gel picture showing the amplification patterns of the 18 *Meloidogyne* isolates using primer-pairs specific to (a) *M. exigua*, (b) *M. incognita* and (c) *M. paranaensis*. *M-1* to *M-18* correspond to isolate codes given in Table 1 of **paper II**.

Amplification of the partial sequence of the 18S nuclear rDNA region yielded a 902 bp band for both *M. exigua* and *M. incognita*. The amplification of the 28S rDNA region yielded a fragment of 342 bp for *M. exigua* and 334 bp for *M. incognita*. When the sequences of the 18S rDNA region from *M. exigua* from this study (HQ709101) were compared with sequences of *M. exigua* retrieved from the GenBank (AF442200 and AY942627) the mean sequence identity was 98.2% and the mean sequence identity between our *M. exigua* (HQ709101) and *M. incognita* sequences from the GenBank was 96.3%. The sequence identity of *M. incognita* from this study (HQ709102) and *M. incognita* from the GenBank was 99.2% whereas our *M. incognita* (HQ709102) and *M. exigua* sequences from the GenBank had a sequence identity of 96.9%. The analysis of the partial sequences of 18S and 28S rDNA genes revealed that *M. exigua* and *M. incognita* from our study are single haplotypes, despite of their variability in perineal patterns (**Table 2 and 3**).

31

Table 2. Sequence identity between sequences generated in this study and sequences retrieved from the GenBank, in M. exigua and M. incognita.

Gene	Species	Country of origin	Host	Accession number	M. exigua[a]	M. incognita[a]
18S rDNA	*M. exigua*[a]	Nicaragua	Coffee	HQ709101	-	0.966
	M. exigua[b]	UK	Coffee	AF442200	0.984	0.959
	M. exigua[b]	Brazil	Coffee	AY942627	0.979	0.983
	Mean				0.982	0.969
	M. incognita[a]	Nicaragua	Coffee	HQ709102	0.966	ID
	M. incognita[b]	Brazil	Coffee	AY942624	0.964	0.997
	M. incognita[b]	Netherlands	ng	EU669939	0.963	0.996
	M. incognita[b]	Netherlands	ng	EU669940	0.960	0.993
	M. incognita[b]	Netherlands	ng	AY284621	0.960	0.992
	M. incognita[b]	ng	ng	AY268120	0.960	0.990
	M. incognita[b]	USA	Soybean	AF535868	0.971	0.985
	Mean				0.963	0.992
28S rDNA	*M. exigua*[a]	Nicaragua	Coffee	HQ709103	-	0.929
	M. exigua[b]	Brazil	Coffee	AF435796	0.995	0.933
	M. exigua[b]	Brazil	Rubber tree	AF435795	0.990	0.938
	M. exigua[b]	UK	Coffee	AF435804	0.990	0.929
	Mean				0.992	0.932
	M. incognita[a]	Nicaragua	Coffee	HQ709104	0.929	-
	M. incognita[b]	Korea	Ng	GQ258784, 6-9	0.929	1.000
	M. incognita[b]	USA	Ng	AY355416	0.929	1.000
	M. incognita[b]	USA	Ng	AY355411,3	0.929	1.000
	M. incognita[b]	USA	Soybean	AF435794	0.924	0.995
	M. incognita[b]	USA	Ng	AY355414,7	0.924	0.937
	M. incognita[b]	USA	Ng	AY355412	0.905	0.928
	Mean				0.926	0.983

Ng = not given; [a]sequences generated in this study; [b]sequences retrieved from GenBank

For 28S rDNA, the *M. incognita* haplotype in our study is the same as those reported by Chen *et al*. (2003) from USA and Oh *et al*. (2009) from Korea. In the case of 18S rDNA the haplotypes for both species are different from those retrieved from the GenBank the same was observed for 28S rDNA from *M. exigua*. *M. exigua* 28S rDNA sequences from our study revealed more similarity to the sequence of one isolate from coffee in Brazil (AF435796) than the isolate from rubber trees in Brazil (AF435795) and coffee from UK

(AF435804). The 28S rDNA sequence of *M. incognita* from the present study was identical to three accessions from USA and five accessions from Korea, but had a sequence identity of only 92.8% with another accession from USA. A phylogenetic analysis by De Ley *et al*. (2002) on RKN showed that *M.incognita* is closely related to *M. javanica* and *M. arenaria*. An explanation can be that *M. incognita* shares obligatory mitotic parthenogenesis with these species (Chen *et al*., 2003; Oh *et al*., 2009) whereas *M. exigua* is closely related to *M. graminicola* and *M. chitwoodi*, which shares meiotic parthenogenesis (Tigano *et al*., 2005). The use of only the 18S and 28S rDNA region for species identification is not a reliable approach and may lead to misidentification of the species. Nevertheless, the 28S rDNA region can be used to identify *M. exigua*, as the 99.2% sequence identity between *M. exigua* haplotypes was significantly higher than their sequence identity with other *Meloidogyne* species (**Table 2 and 3**).

Comparisons between the 18S rDNA sequence of *M. exigua* from our study and *M. exigua* from the GenBank, revealed that our *M. exigua* has a unique variable site within the 18S rDNA at the 196th nucleotide. At this position *M. exigua* has a *T* instead of *A* or *indel*. In *M. incognita* there was no such unique mutation. The sequence similarity between *M. exigua* from our study and other *M. exigua* sequences is of the same level as the similarity between *M. exigua* and *M. oryzae*, *M. graminicola* and *M. fallax*. Comparisons of the 28S rDNA sequences of *M. exigua* to the same species revealed that *M. exigua* has three unique variable sites at the positions 26, 31 and 55. These sites are *T vs G/A*, *T vs C* and *T vs G/indel*, respectively. There was no such mutation in *M. incognita*. *M. exigua* has unique variable sites within both the 18S and 28S rDNA regions when compared with other *Meloidogyne* species, including those that share similar reproductive mechanisms. The unique variable sites in *M. exigua* can be used to develop new species-specific SCAR markers for identifications of this species. The 18S rDNA have been useful in RKN phylogenetic studies, but there are some groups of species that are not well resolved (Page & Holmes, 1998). Our results have demonstrated the presence of *M. exigua* and *M. incognita* in the eighteen isolates collected from the two coffee growing regions of Nicaragua.

Table 3. *Mean sequence identity matrix between sequences from the two Nicaraguan Meloidogyne species (columns) identified in this study and sequences of the same species and other closely related Meloidogyne species retrieved from GenBank (rows).*

Species sequence from GenBank	*M. exigua* from Nicaragua			*M. incognita* from Nicaragua		
	18S	28s	Mean of 18S and 28S	18S	28s	Mean of 18S and 28S
M. exigua	0.982	0.992	0.987	0.969	0.932	0.951
M. oryzae	0.985	-	-	0.959	-	-
M. graminicola	0.983	0.948	0.966	0.957	0.937	0.947
M. fallax	0.982	0.920	0.951	0.963	0.914	0.939
M. incognita	0.963	0.926	0.945	0.992	0.983	0.988
M. paranaensis	0.966	0.929	0.948	0.993	1.000	0.997
M. javanica	0.963	0.929	0.946	0.995	0.995	0.995
M. hispanica	0.963	0.929	0.946	0.993	0.980	0.987
M. arenaria	0.962	0.918	0.940	0.994	0.982	0.988
M. floridensis	0.964	-	-	0.997	-	-
M. morocciensis	0.964	-	-	0.997	-	-
M. ethiopica	0.965	-	-	0.996	-	-
M. arabicida	0.964	-	-	0.993	-	-
M. chitwoodi	0.980	0.893	0.937	0.961	0.869	0.915
M. konaensis	-	0.929	-	-	0.995	-

5.3 Analysis of genetic diversity in *Meloidogyne exigua* in Nicaragua coffee plantations by using ISSR (Paper III).

Of the 12 ISSR primers used in this study, 89.6% were polymorphic and three (ISSR-835, ISSR-880 and ISSR-891) were 100% polymorphic. The primer ISSR-891 revealed the highest genetic diversity with a Shannon diversity index (I_L) of 0.61 and Nei´s gene diversity (H_L) of 0.42. Previous studies have shown a similarly high genetic polymorphism in different *Meloidogyne* species. Randing *et al*. (2002) observed 67.5% in populations of *M. arenaria, M. exigua* and *M. hapla* using RAPD markers. In contrast, Carneiro *et al*. (2004) observed only 8.6% genetic diversity between two isolates of *M. exigua* from coffee farms in Brazil, using RAPD markers. In our study, the level of polymorphism between the two Matagalpa populations (18%) was significantly

lower when compared to the whole populations studied (89%). According to Andersson *et al*. (1998) different molecular markers may reveal different patterns of genetic structures within plant-parasitic nematodes.

The overall mean estimates of Shannon diversity (I_P), Nei´s gene diversity (H_P) and percent of polymorphic loci (%PL_P) for the 18 *M. exigua* populations were 0.45, 0.30 and 88.7 respectively. The diversity estimates for the populations from each region were lower compared to the whole study area. For example, I_P, H_P and %PL_P were 0.44, 0.29 and 88.9, in that order, for the 15 South Pacific populations and were 0.42, 0.28 and 81.5 respectively, for the eight Masatepe populations. The lowest genetic diversity was obtained for the two Matagalpa populations with I_P = 0.11, H_P = 0.08 and % PL_P = 18.2. The level of the genetic diversity in the present study was significantly correlated with the number of populations analyzed. This result suggests the presence of a unique genetic variation within each population and the level of genetic diversity of the nation-wide *M. exigua* population in Nicaraguan coffee plantations is expected to be higher than reported in this study. Although the number of populations included in this study is rather low, the pattern of genetic variation obtained suggests similar pattern and extend of genetic variation in other populations in the country.

AMOVA revealed no significant differences among regions and provinces when all populations were considered (P≥0.05). However, slightly significant differentiation was obtained (F_{ST}= 2.7; P≤0.03) among Masatepe, Carazo and Matagalpa. The AMOVA analysis among and within regions showed that 97.3% of the total genetic variation was due to variation within regions and only 2.7% of total variation was accounted for variation between regions. The results suggest that good genotypic and phenotypic analysis of a limited number of *M. exigua* populations can help to understand the general characteristic of the populations and that similar pest control approaches can easily be implemented throughout geographical areas, especially when introducing new varieties. From an evolutionary perspective, the lack of high differentiation between populations may also suggest that the nematodes were introduced to Nicaragua not too long time ago and/or that the origin of the *M. exigua* populations in Nicaragua may be the same.

The mean of Jaccard´s genetic similarity among 18 *M. exigua* populations was 0.51 whereas that between *M. exigua* (all populations, except M-16) and *M. incognita* (M-16) was 0.42. The Jaccard´s similarity coefficient between *M. exigua* populations ranged from 0.40 to 0.67. The highest genetic similarity was between the two populations from Matagalpa (M-7 and M-8) and from Granada and Masatepe (M-3 and M-4) and the lowest was between populations

from Masatepe (M-1) and from Masaya (M-15). The population M-3 (from Granada) and M-4 (from Masatepe) also showed relatively high genetic similarity. Carpenter *et al*. (1992) reported a high level of variation in populations of *M. exigua* from geographically closely linked sites which are in agreement with our results for the M-7 and M-8 populations. Our results suggest that adverse conditions may lead to an increase in the genetic variation of the nematode population. The sex determination in parthenogenetic root-knot nematodes is epigenetic and under strong influence of adverse environmental factors, juveniles can develop into males under certain conditions (Triantaphyllou, 1973). *M. exigua*, among other parthenogenetic RKN, can reproduce both by cross-fertlization and by meiotic parthenogenesis (Castagnone-Sereno, 2002). More variability is expected in fields with *M. exigua* when the reproduction mode is facultative meiotic parthenogenesis rather than mitotic parthenogenesis (Triantaphyllou, 1985).

The cluster analysis revealed a clear separation of *M. exigua* from *M. incognita*. The *M. exigua* populations were grouped into six clusters with a mean Jaccard´s similarity coefficient of 0.50. Cluster I (M-1), II (M-15) and VI (M-5) consists of single populations. Cluster III was the largest with eigth populations of which four were from Masatepe and a sub-cluster with two populations, M-7 and M-8, and 95%, bootstrap support. This cluster also contains a sub-cluster of the M-3 and M-4 populations from Granada and Masatepe, respectively, with a 76% bootstrap support. In cluster IV M-18 and M-19 clustered together with relatively low bootstrap support (53%). Four populations from three provinces were clustered under cluster V, with M-11 and M-12 in one sub-cluster and M-13 and M-14 forming the second sub-cluster. In general, the populations were clustered independent of geographic origin. We could observe that populations from the same province were clustered together (M-7 and M-8) only at one occasion. We also observed that the population from Dipilto (M-11) from the North Central region clustered together with a population from the Carazo province (M-12) from the South Pacific region with high bootstrap support (**Fig. 3**). Devran *et al*. (2008) also reported the absence of correlation between the level of genetic similarity and geographic origin in three *Meloidogyne* species (*M. arenaria, M. javanica* and *M. incognita*). The results of the present study suggest the absence of significant geographical differentiation of the nematodes and thus similar management practices may be effective throughout the areas where the nematodes are found in the country.
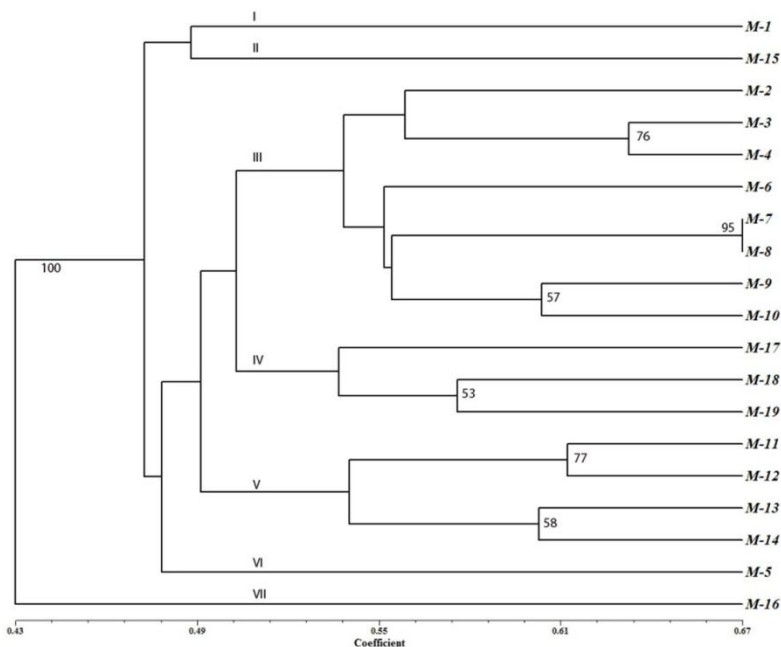
Figure 3. Consensus dendrogram of *Meloidogyne exigua* and *M. incognita* populations generated based on Jaccard's similarity coefficient. Bootstrap values based on 1000 replicates are given in each node. Population codes are given in Table 1 in paper III.

5.4 Genetic diversity of arabica coffee (*Coffea arabica* L.) in Nicaragua as estimated by microsatellite markers (Paper IV).

The overall gene diversity estimated as H_T *was* 0.353 whereas the overall within-population variation estimated as H_S was 0.291. H_{Loci} for each population across the eight polymorphic loci ranged from 0.23 (population CT4) to 0.47 (population B2) whereas percent polymorphic loci *(%PL)* ranged from 0.33 to 0.58. When the populations were grouped into varieties, the mean Nei's gene diversity ranged from 0.24 (cv. Maracaturra) to 0.37 (cv. Catimor) with corresponding lowest and highest *%PL* of 0.33 and 0.52. Among the eight coffee varieties included in this study Catimor showed the highest gene diversity with a mean genetic diversity of 0.37 and percent of polymorphic loci of 0.52. This variety is interesting not only due to its high diversity but also due to the fact that it contained populations (CM2 and CM3), which were significantly different from all other populations included in the present study. For example, the alleles in CM2 and CM3 at the SSR locus 471 were different from all other populations except in CM4. The higher diversity in this variety

is not surprising as it was developed from crosses of *C. arabica* and *C. canephora*. *C. canephora* has higher genetic diversity than *C. arabica* as shown in several studies (Moncada & McCouch, 2004; Cubry *et al*., 2008).

Population specific alleles were detected in five populations from four different varieties. Population B2 has a unique allele (89 bp) whereas populations CA3 and CM4 shared a unique allele (97 bp) at locus Sat207. Similarly population B3 and CT6 have unique alleles at SSR03 and CaM35 loci. Sat207 has two rare alleles (ca 89 bp and 97 bp) in addition two dominant alleles (ca 82 bp and 93 bp). The 89 bp allele was obtained in only one population whereas the 97 bp allele was obtained in two populations. Taking into consideration the amphidiploids nature of *C.arabica*, it is most likely that 82 bp allele was originated from one of the two progenitors whereas the other three alleles were originated from the other progenitor of *C. arabica* (**Fig.4**).
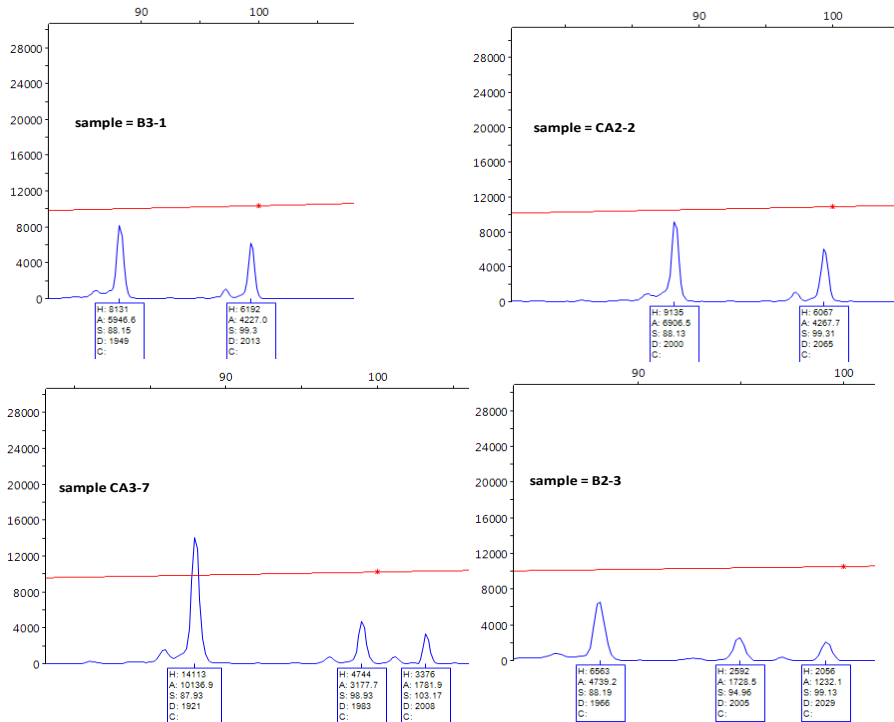

Figure 4. The electrophoretogram showing the alleles at the SSR locus *Sat207*.

Eight of 12 investigated loci, were polymorphic. The loci with the highest gene diversity were CM5, Sat207 and CaM03 with H_T of 0.50, 0.50 and 0.54, respectively. The level of differentiation between populations varies significantly among polymorphic loci. Sat235 was monomorphic across all 26 populations. Sat207 and Sat235 were reported to be linked tightly to the locus (*Ck-1*) that carry a major gene conferring resistance to the coffee berry disease (CBD) (Gichuru *et al*., 2008). Since the resistance gene *Ck-1* was introduced to *C. arabica* from *C. canephora* (Gichuru *et al*., 2008), it is likely that the latter three alleles in *C. arabica* might correspond to the *C. canephora* ancestral genome. Another interesting locus is CM5, two alleles were detected at this locus and all 260 individuals studied were heterozygous for these alleles. Several authors have indicated that the cross-species transferability of SSR markers including EST-SSRs within the genus *Coffea* is high (Baruah *et al*., 2003; Poncet *et al*., 2007; Aggarwal *et al*., 2007). Given the fact that *C. arabica* is a self-pollinating species, the 100% heterozygosity obtained at this locus can only be explained by its amphidiploid nature.

The cluster analysis revealed five clusters supported by moderate to high bootstrap values. Cluster I contained two populations of cv. Catimor (CM2 and CM3) with a bootstrap value of 100%. Cluster II contained three populations (CM4, B2 and CT2), all of which belonged to different varieties with a 98% bootstrap support. Similarly, cluster III contained two populations (CR2 and P) which belonged to cvs Catuai rojo and Pacas, respectively, with a 70% bootstrap support. Cluster IV contained four accessions (CM1, CT5, M and PA) all of which belong to different varieties with a 64% bootstrap support. Cluster V is the largest cluster that contained 15 of the 26 accessions that were further grouped into two sub-clusters and one solitary population B3 (**Fig. 5**). Generally, the clustering of populations according to variety was low. Cluster analysis of the 26 *C. arabica* populations revealed that, in most cases, the clustering pattern of the populations was not in line with varietal classification of the populations. When the autogamous nature of the crop is considered, such poor clustering pattern of the varieties is somewhat surprising. However, the obtained results can be due to several reasons: (1) each variety might be the result of crosses between different genotypes that belong to the same old variety; (2) some degree of gene flow between varieties through cross pollination, as more than one variety exist in the same plantation; and (3) some of the populations might have been misclassified at different generations since the development of the varieties.

The first three principal coordinates explained 77% of the variation among the 260 individual plants that were analyzed using PCoA. The analysis showed

that the weak grouping of the accessions according to variety was mainly due to the high variation within populations. This analysis revealed three major clusters of individuals. However, some individuals from various populations and varieties stood alone across the four quadrants. In quadrant I, B2-10; in quadrant II, B2-1, B5-5, and CM3-10; in quadrant III, CT2-7 and CT5-2; and in quadrant IV, PA-8 was clearly separated from the other individuals. However, the spreading of the individuals across the four quadrants shows some level of genetic variation.

AMOVA revealed that the observed genetic variation among populations is highly significant (P<0.0001). Similarly, the differentiation among different coffee varieties was significant ($F_{CT}=0.08$; P=0.023) contributing to 7.9% of the total variation. The presence of unique alleles in four of the eight varieties contributed to the significant variation obtained. The most differentiated populations were CM2, CM3 and CM4, which all belong to cv. Catimor. CM2 and CM3 were significantly differentiated from all populations whereas CM4 was significantly differentiated from all population except B2. CA5 was the least differentiated population being significantly differentiated from only four populations.
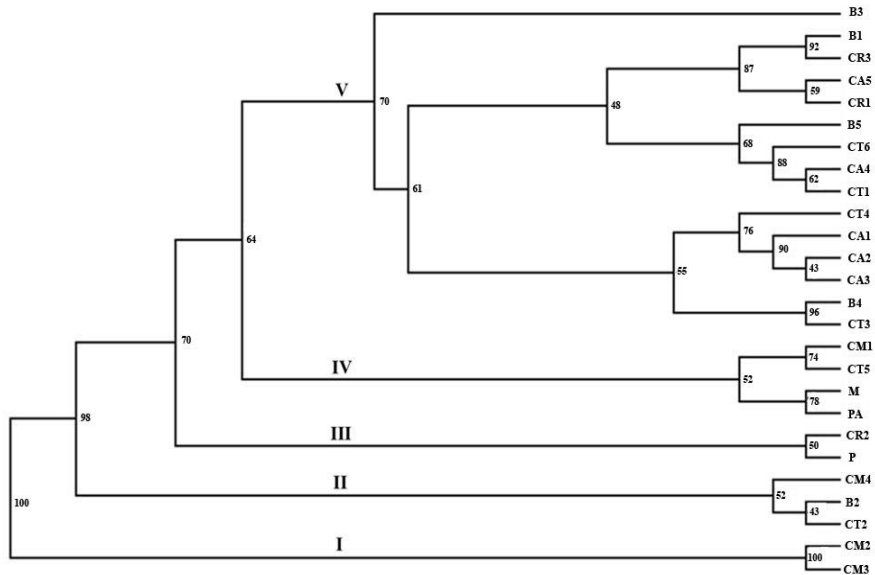

*Figure 5.*SSR based UPGMA phenogram for the 26 coffee populations based on Nei's standard genetic distance. Numbers in front of the branches are bootstrap values.

6 Conclusions, recommendations and future prospects

6.1 Conclusions

1. The management system had a distinct effect on the occurrence of *Meloidogyne* spp. and *Pratylenchus* spp. Higher populations of *Meloidogyne* occurred in conventional management with no shade and with shade from nitrogen fixer species while higher populations of *Pratylenchus* spp. occurred in both conventional and organic management but shaded with non fixer nitrogen species.

2. The use of perineal pattern, SCAR markers and partial sequences of the 18S and 28S rDNA region in the analysis of eighteen isolates of *Meloidogyne* species collected from coffee roots across Nicaraguan coffee plantations revealed the presence of *M. exigua* and *M. incognita.*

3. The partial sequences of the 18S and 28S rDNA regions showed that *M. exigua* and *M. incognita* are comprised of single haplotypes.

4. *M. exigua* has unique variable sites within the 18S and 28S rDNa regions unlike other *Meloidogyne* species, including those sharing a similar mode of reproduction.

5. *M. exigua* showed high genetic diversity within each population, and the level of genetic diversity of the nation-wide is expected to be higher than the reported in this study.

6. There were significant genetic differences between coffee varieties and the highest genetic diversity was recorded in cv. Catimor.

6.2 Recommendations and future prospects

The results obtained in this study provide information for a better understanding of the occurrence of plant-parasitic nematodes in Nicaraguan coffee under different management systems, the predominant species of RKN and their genetic diversity. This study also provides basic information about the genetic diversity present in the main arabica coffee-varieties used.

The distinct effect of management systems on *Meloidogyne* spp. and *Pratylenchus* spp. populations found in this study, suggest that controlled experiments should be conducted to study the mechanisms involved in the occurrence of plant-parasitic nematodes. Since the occurrence of plant-parasitic nematodes in the different coffee management systems seems to be influenced by the type of shade species, the role of shade species on nematode populations should be further studied to investigate whether the species used to shade coffee might be favour plant-parasitic nematodes.

Based on the results that demonstrate the presence of both *M. exigua* and *M. incognita*, and the predominance and wider distribution of *M. exigua*, studies should be carried out to confirm the presence, predominance and geographical distribution of both species in different coffee growing regions in Nicaragua.

The management systems and the agro-ecological conditions are variable in Nicaragua and a comprehensive study is needed to investigate the influence of the management systems on the genetic diversity of RKN. Considering that our results suggest that the analysis of a limited number a of *Meloidogyne* species can help to understand the characteristics of the nematode populations, an extensive study about the genetic diversity present in the plant-parasitic nematodes distributed in the different coffee growing regions of Nicaragua is needed, especially when pest control and new coffee varieties are planned to be introduced.

Further studies are needed on the genetic diversity of the Nicaraguan coffee varieties in order to find resistant or tolerant genotypes in relation to pest and diseases, particularly the RKN.

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Nematodos Noduladores y Café en Nicaragua: Sistemas de Manejo de Café, Identificación de Species y Estudio de la Diversidad Genética de *Meloidogyne exigua* y de Variedades de Café.

Resumen

El cultivo del café (*Coffee arabica* L.), representa una de las actividades de mayor importancia en la economía de Nicaragua. Dicho cultivo se ve afectado por diversos factores adversos que limitan la producción, entre los que destacan los nematodos fitoparásitos, los cuales son considerados uno de los mayores factores que afectan la producción de café hasta en un 15%.

El propósito de esta tesis es contribuir al conocimiento de los nematodos fitoparásitos del café mediante la evaluación del efecto de sistemas de manejo, la identificación de especies y el estudio de su diversidad, así como el estudio de la diversidad genética de variedades de café arábigo. Esta tesis incluye estudios sobre la incidencia de *Meloidogyne* spp. y *Pratylenchus* spp. en diferentes sistemas de manejo de café, identificación de nematodos noduladores provenientes de 18 fincas de dos regiones cafetaleras de Nicaragua, también se estudio la diversidad genética de *Meloidogyne exigua*, así como la diversidad genética de las principales variedades de café .

Los resultados indican que *Meloidogyne* spp. y *Pratylenchu*s spp. se presentaron en todos los sistemas de manejo evaluados. Las poblaciones de *Meloidogyne* spp. fueron más altas en manejo convencional a pleno sol y en manejo convencional con sombra de especies de leguminosas. En el caso de *Pratylenchus* spp. sus poblaciones fueron más altas tanto en manejo convencional y manejo orgánico con sombra de especies no leguminosas. Las especies de nematodos noduladores identificados fueron *M. exigua* en todos los aislados y *M. incognita* en solamente uno de los aislados de café, este estudio además reveló la predominancia de *M. exigua* en todas las muestras analizadas. La secuencia parcial de la región 18S y 28S indicó para el caso de *M. exigua* la presencia de sitios únicos variables en la secuencia de ADN, los cuales fueron diferentes inclusive en las especies de *Meloidogyne* que comparten el mismo mecanismo de reproducción, este hallazgo podría ser de gran utilidad en el diagnóstico de esta especie. El uso de ISSR, reveló mayor diversidad genética de *M. exigua* entre las poblaciones obtenidas de la misma localidad que entre poblaciones de diferentes localidades y que entre poblaciones de diferente región geográfica. Este resultado podría indicar que la diversidad de esta especie podría ser mayor a nivel nacional. Finalmente, el análisis de la diversidad genética de las variedades de café arábigo, mostró que la mayor variabilidad genética se registró en la variedad Catimor, lo que podría ser de gran utilidad en el desarrollo de programas de mejoramiento genético de variedades de café enfocado al manejo de problemas fitosanitarios, principalmente nematodos fitoparásitos asociados al café en Nicaragua.

Palabras claves: Café arábigo, diversidad genética, ISSR, nematodos noduladores, manejo de café, variedades, SSR, marcadores SCAR, región ribosomal de ADN 18S y 28S.