INFLUENCE OF CLIMATIC VARIABLES AND BIOLOGICAL CONTROL AGENTS (NEMATODES) ON THE DISTRIBUTION AND SURVIVAL OF THE COFFEE BERRY BORER, *HYPOTHENEMUS HAMPEI*, IN PUERTO RICO

By

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ABSTRACT

Coffee is the second largest export product of developing countries and is one of the ten most important cultivated crops in Puerto Rico, especially in the mountainous region. The coffee berry borer (CBB) *Hypothenemus hampei* Ferrari (1967), which arrived in Puerto Rico in 2007, is considered the most destructive pest in all coffee producing areas worldwide. The control of CBB is an enormous challenge because this insect spends most of its life inside the coffee berry, even, the use of insecticides has proved ineffective. Therefore, the development and implementation of biological control alternatives, as part of integrated pest management (IPM), is very important for the sustainability of the coffee industry. In this dissertation, I combine the spatial distribution of suitable habitats for CBB, detection of entomopathogenic nematodes (EPNs), a potential biocontrol agent and determining soil factors that affect the natural occurrence of EPNs in any given site. Species distribution model models (Chapter II) was performed using a total of 97 (241 sites sampled) georeferenced CCB presence and nineteen bioclimatic variables and altitude. Distribution maps were generated illustrating the suitable area for coffee berry borer in Puerto Rico. CBB distribution is favored by precipitation of wettest quarter, highest altitude and precipitation seasonality. In order to validate the model result, field percent of infestation was also calculated by the total of borer berries divided on total of berries in selected 3 branches in each sample site and positive relationship between model suitable index and field infestation of CBB was found. Furthermore, we sampled a total of 32 farms throughout the coffee production area in Puerto Rico using the insect bait method to extract EPNs (Chapter III). A total of 143 EPN isolates were recovered. I also made the first

report of the presence of the recently recognized as an EPN *Oscheius myriophila* which was identified, based on molecular and morphometrical analysis, in 90.85% of the sample site. Finally, each nematode sampled site was characterized, firstly, according to coffee agroecosystem (full sun or under shade) and second, by soil physicochemical characteristics (Chapter IV). Using a generalized linear model (GLM), I determine the interaction of soil parameters such as pH, texture and elevation coupled with shade coffee to predict *O. myriophila* occurrence. In conclusion, dissertation provides a basic framework to develop integrated pest management for CBB integrating better knowledge about its preferences in climatic condition in order to prevent outbreak and the use of native EPN *O. myriophila*.

DEDICATION

I dedicate my dissertation work to my wife Yesely Jimenez and my son Joshua García for being my inspiration and the reason for not giving up in the most difficult moments.

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Chapter I

GENERAL INTRODUCTION

1.1. Introduction

Coffee is the main export product of many developing countries (DaMatta et al., 2010) and is the second-most globally traded commodity after oil (Davis et al., 2012). Botanical evidence suggests Ethiopia as origin (Mussatto et. al., 2011) and have been described nearly 80–100 species (Dias and Benassi, 2015), but only 2, *Coffea arabica* and *Coffea canephora* (Robusta), are considered in trade (Teketay, 1999). These species represent approximately 99% of global coffee production (Jayakumar et al., 2017), furthermore, worldwide consumption is 70% for *Coffea arabica* and 30% for *C. canephora* (DaMatta et. al., 2007). Coffee was introduced to Puerto Rico in 1736 and established as an industry in the early 1880s (Bergad, 1978), by the end of the 19th century it turned in one of the main commercial crops, reaching highest price in the world (Dietz, 1986). In the beginning, the coffee was cultivated primarily under shade condition (Pumarada, 1989), then, in order to increase yield, the intensive full sun techniques were encouraged (Borkhataria et al., 2012). Regardless of the type of growth system, coffee trees are perennial evergreen, this makes it attractive for arthropods (Barrera, 2008). More than 850 species of insects have been reported to feed on the coffee tree (Le Pelley, 1973), of which, hardly thirty species cause economic losses (Cristancho et. al., 2015).

1.2. Coffee Berry Borer

The coffee production in Puerto Rico faces the Coffee Berry Borer (CBB) *Hypothenemus hampei* Ferrari (1967) (NAPPO, 2007), considered as a most destructive biotic threat for coffee production (Soto-Pinto et. al., 2002). A fertilized female lays 2 or 3 eggs per day for several weeks, which results in 30 to 70 progenies in a single coffee fruit and the larvae begin to eat from the endosperm immediately after hatching (Bergamin, 1943). Some of

the main damages caused by this pest are the fall of young fruits, destruction of the berry, weight and quality reduction (Gómez et. al., 2015).

The CBB spends most of its life cycle inside the coffee berry, only the adult female can fly and infect another berry (Vega et al., 2015). Due to its cryptic habit, it is an extremely difficult pest to control, even management with agrochemicals have not been effective (Vega et al., 2009). Additionally, agrochemicals have been reported associated with animal and human health implications, as well as the damage to the environment (Segal and Glazer, 2000). Another disadvantage is the resistance of the CBB towards synthetic chemical pesticides (Gongora et al., 2001).

1.3. Biological Control

In order to reduce CBB populations, the development and implementation of biological control alternatives, as part of integrated pest management (IPM), is very important for the sustainability of the coffee industry (Aristizábal et al., 2016). Biological control is defined as control or regulation of pest populations at innocuous densities using natural enemies such as predators, parasitoids and pathogens (Hawkins and Cornell., 2008). Thus, is an alternative to using chemical insecticides (Pimentel, 1991). An organism is considered natural enemy when feeds on another organism (Smith and Capinera, 2017). Two main approaches of biological control are proposed for CBB management, first, introduction of exotic enemies (classical biocontrol) and second, protection and enhancement of native biocontrol agents (conservation biocontrol) (Eilenberg et al., 2001).

1.4. Species Distribution Model

The successful implementation of a biological control strategy requires a thorough understanding of the relationship with the agroecosystem of the target population, particularly its spatial distribution (Escobar-Ramírez et. al., 2019). Many statistical approaches are available to model species distribution such as GLM, GAM, BRT, BIOCLIM, CLIMEX, GARP and MaxEnt (Bellard et al., 2012), The development and application of this have seen a rapid increase over the last decade (Radosavljevic and Anderson 2014). Within that, maximum entropy algorithm (MaxEnt), is an excellent choice for presence-only data (Elith and Leathwick 2009). Species distribution models (SDM) are cartographic representations of the suitability space for the presence of a species based on the variables used to generate such representation (Peterson, 2006), revealing species ecological requirements and predictive variables as well as the importance of each variable in model building (Araújo and Guisan, 2006). SDM is defined as a simplified representation of reality that reflects some of its properties (Guisan et al., 2007). SDM is useful for understanding the impacts of climate on habitat suitability (Austin, 2007). For CBB, models indicate that in any of the CO_2 emission scenarios, CBB would expand its distribution area reaching 77.8% of Arabica coffee production areas compared to the current 57% (Magrach and Ghazoul, 2015) and as well as its severity (Jaramillo et. al., 2010). Predicting and understanding the change in distribution and behavior, at local level, can help in the design strategies to prevent pest outbreak.

1.5. Entomopathogenic Nematodes

Entomopathogenic nematodes (EPN) are used globally as safe biocontrol agents against soil-borne insect pests (Ehlers, 2005). This microscopic organism living symbiotically with pathogenic bacteria in order to kill the parasitized insects within 24-48 hours (Denno et al., 2008). EPNs have been tested and proved that there is either no risk to warm-blooded animals or to plants (Boemare et al., 1996). Entomopathogens differs from parasitism in the use of virulent pathogenic bacteria against the insect host (Dillman et al., 2012). The relationship between EPNs and associated bacteria is considered as a mutualist because the bacteria cannot penetrate inside the insect without the nematode and the latter cannot grow and reproduce in the absence of the bacteria (Poinar and Grewal, 2012). Furthermore, the EPNs protects its bacteria from environmental elements (Poinar, 1990), while, the bacteria contribute to degrading the insect cadaver into nutrients (Forst and Clarke, 2002) and protect the cadaver from opportunists (Baur et. al., 1998). Traditionally, these characteristics mainly referred to nematodes of the families *Steinernematidae* and *Heterorhabditidae*, but recently, species of the genus *Oscheius* have been considered (Dillman et al., 2012). Also, *Heterorhabditidoides* was described as an EPN genus (Zhang et al., 2008), but, based on morphological similarity was unified with genus *Oscheius* (Torres-Barragan et al. 2010; Liu et al. 2012).The associated symbiotic bacteria for *Steinernematid* is *Xenorhabdus* spp., for *Heterorhabditids* is *Photorhabdus* spp. and *Oscheius* mainly is *Serratia* (Lewis and Clarke, 2012; Zhang, 2008).

The free-living infective juvenile (IJ) is only stage adapted for survival in the environment out of corpse of the insect (Poinar, 1990). This adaptation includes lack of feeding, closed external openings (mouth and anus) and two layers of external membrane cuticle that

provides additional protection and prevents water loss (Glazer, 2002). The IJ host finding tactics is classified as cruisers (actively searching), ambushers (more sedentary and waiting for hosts) and intermediate foragers (Grewal et al., 1994). The IJ penetrates the insect host through the mouth, anus, spiracles and also have the facility to penetrate through the cuticle of the insect (Kaya and Gaugler, 1993) and once inside they release the symbiotic bacteria (Goodrich-Blair and Clarke, 2007). A single IJ is sufficient to develop the infection (Forst and Clarke, 2002) and after 7 to 10 days a new generation of IJs is produced that go out to look for new prey (Wang et. al., 2007). The IJ carried the symbiotic bacteria in a receptacle in the anterior part of the intestine for S*teinernematids* and in the gut mucosa for *Heterorhabditids* (Ciche and Ensign, 2003; Martens and Goodrich-Blair, 2005). Main attributes of the EPNs include a wide host spectrum, active host seeking, killing the host within 48 h, easy mass production, long-term efficacy, easy application, and are environmentally safe (Shapiro-Ilan et al., 2017).

EPNs which naturally occur in the soil (Hominick et. al., 1996), are recognized as a potential inundative biological control agent (large numbers of natural enemies are released with the expectation that these organisms will effect immediate control) for several agriculture important insect pests of different orders (Divya and Sankar, 2009), including insects in cryptic habitats such as the soil and inside plant parts (Kopenhoffer et al., 2007). EPNs have been applied successfully against both soil inhabiting and above-ground insects (Jagdale et al., 2009; Shapiro-Ilan et al., 2006). In recent years with the use of certain adjuvants that can enhance efficacy, interest has been increasing in foliar application (Schroer et al., 2005) because JIs need to be able to survive desiccation prior to infect a host (Arthurs et al., 2004).

Several nematodes species have been reported as a successful biocontrol agent for CBB with mortalities above 80% (Lara et. al. 2004; Manton et. al., 2012), specially of 12.4 % of CBB population that remains in the soil at the end of the harvest (Moreno-Valencia et. al., 2001). Also, adults and larvae can be infected (Allard and Moore, 1989). The EPN's pathogenicity may vary among genera, species and even strains of the same species (Lewis et. al., 2006), therefore, is essential for coffee researchers to sample coffee growing areas throughout the world for the presence of other nematodes infecting the coffee berry borer (Vega et. al., 2009).

1.6. Environmental factors affecting nematode-insect interactions

The success of using of EPNs to control the target pest depends on a variety of biotic and abiotic factors that affect its persistence and survival in the soil (Shapiro-Ilan et al., 2012). The most critical abiotic factors are soil texture, moisture, temperature, pH, altitude and soil organic matter and biotic such as presence of susceptible hosts, competitors and natural enemies (Campbell et al., 2003; Glazer, 2002; Shapiro-Ilan et al., 2012; Stuart et al., 2015). Environmental condition can also interfere with the infection process of the EPNs to insect host (Susurluk et al., 2004). Generally, soils with higher content of clay restrict IJs movement which can result in reduced nematodes survival and efficacy (Nyasani et al., 2008). Consequently, is essential to understand these interactions in order to enhance the potential of EPN as biocontrol agents in a particular soil type (Jaffuel et al., 2016).

1.7. EPNs, native vs exotic

The optimum biocontrol strain will depend not only on the host but also the conditions under which the control agent will need to be active. Therefore, is preferable to use native species for biocontrol agents because they are already adapted to the environment

(Dolinski et al., 2017; Shields and Testa, 2015), and to avoid many of the risks associated with introducing exotic species (Abate et al., 2017). Some risks of the introduction of exotic EPNs are that they may induce exclusion of local species and they may be inefficient towards local insect pests as they may be less well-adapted to local environmental conditions (Miller and Barbercheck, 2001). Native EPNs have also been recommended for the control of invasive pests (like CBB in Puerto Rico) (Vincent et al., 2007).

1.8. Structure of this Dissertation

The general aims of this dissertation are to identify the climatic variables that most influence the distribution and survival of the coffee berry borer in Puerto Rico, as well as determine the presence of potential biocontrol agents such as EPNs in coffee growing areas and the factors that affect the survival and distribution of this EPN. This will help provide answers to questions such as: What are the climatic variables that most affect the distribution and survival of the CBB in Puerto Rico? also, are there potential biocontrol agents of the CBB in the ground? This would have a beneficial effect by allowing us to integrate these tools into management programs of this and other pests on the island and many other coffees producing countries and making the coffee industry more profitable and friendly to the environment. The hypothesis under testing is that some abiotic and biotic factors affecting the survival of the CBB. Environmental factors such as temperature, precipitation, have a strong influence on the development and distribution of the CBB. Although the CBB is relatively recent introduction to Puerto Rico, it has become the most abundant pest insect in coffee plantations, this makes CBB as a possible prey for EPNs which are generalists.

In Chapter II, I generated a species distribution model in order to determine the suitable areas for CCB and to understand which main bioclimatic variable drives its distribution. I also tested the relationship between CBB infestation levels and suitability index. In Chapter III, I conducted a wide survey around the main coffee growth area to isolated and identified EPNs. I further examined morphometrical variation between strains by trait comparison of EPNs from different locations. Finally, in Chapter IV, I determined the favorable soil characteristics for native EPNs by correlating the soil physicochemical characteristics and EPNs detection.

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CHAPTER II

Modelling Distribution of Suitable Coffee Berry Borer Habitat in Puerto Rico

Abstract

Coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari), the most serious coffee pest in all producer countries was first reported in Puerto Rico in 2007 and quickly established in all coffee grown zone. Its distribution and damage are determined by several conditions including bioclimatic variables. In this study, the main aim is to model the distribution of suitable CBB habitat in relation to environmental factors such as altitude, temperature and precipitation patterns. MaxEnt model was used to calculate the habitat suitability index for the species by incorporating 19 bioclimatic variables plus elevation along with species detection data. Also, the model analysis provides, the Jackknife test, for the contribution of each variables on the model building. To validate the model, we used historical precipitation and temperature data from 1930 to 2015 to assess the relationship of percentage of infestation and suitability index. The MaxEnt model performed better than randomly expected with an average test Area Under Curve (AUC) value of 0.913 (±0.02) projected highly suitable areas (80-100 %) for all current known areas of CBB presence data. The most contributive variables to create the potential distribution model were: Precipitation of Wettest Quarter (30.56), altitude (22.21) and Precipitation Seasonality (18.33). We found a significant linear relationship (p-value=0.036, R2=0.16) between the suitability for CBB presence index and percent of infestation of CBB. This study provides important information about high suitable habitats and main climatic variables which drives the development, reproduction, and survival of CBB in Puerto Rico coffee growing area.

Key Words: *Hypothenemus hampei,* Maxet, climatic variables, suitable index

2.1. Introduction

The global distribution and abundance of a species is strongly influenced by abiotic factors such as climatic conditions (McDowell et al., 2014). Species distribution model (SDM) permits the projection of the potential distribution of species, based on the relationships among species, bioclimatic variables and their ecological requirements (Elith and Leathwick, 2009). These models help to identify environmental factors that may limit a species distribution (Araújo and Guisan, 2006; Parsa et al., 2012). In agriculture, SDM can help farmers to established pest management strategies of invasive species (Roura et al., 2009), to determine the impact of global climate change on pests (Rodríguez et al., 2007), prioritizing pest control efforts by first targeting highly suitable areas (Kumar, et al., 2015).

MaxEnt algorithm (Phillips et al., 2004) is one of the most useful methods to do SDM using only presence data (Elith, et al., 2011; Merow et al., 2013). MaxEnt model is a maximum entropy-based machine learning program that estimates the probability distribution for a species occurrence based on environmental constraints (Phillips et al., 2004). The model generates an estimate of the probability of presence of the species varying from 0 to 1, where 0 is the lowest and 1 the highest probability (Phillips and Dudík., 2008) and can use to estimate the relative suitability of the habitat currently occupied by the assessed species (Warren and Seifert, 2011).

Insects are highly affected by temperature, moisture, humidity and seasonal variations (Sutherst, 2000). The most damaging insect in all coffee producing countries is the coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae) (Baker, 1999). The distribution and damage caused by this pest are determined by several climatic factors such as altitude, temperature and precipitation patterns (Constantino et

al., 2011; Jaramillo et al., 2011; Rodríguez et al., 2013). In Puerto Rico, CBB was first detected in 2007 (NAPPO, 2007) and rapidly established throughout the coffee producing areas on the island (Mariño, 2017). The infestation levels for Puerto Rico were reported higher than previous reports from other countries (Mariño et al., 2017). The climatic factors that contributed to a fast distribution and successfully established of CBB in the island are still not well documented.

In this study, we used maximum entropy model (MaxEnt) for modeling the CBB to comprehend the characteristics of a suitability habitat for CBB in Puerto Rico, identify bioclimatic variables associated with CBB distribution and the relationship between CBB infestation levels and environmental factors.

2.2. Material and Methods

2.2.1. Occurrence and Environmental Data

Ninety-seven georeferenced coffee farms in Puerto Rico were used to determine presence of *H. hampei*. A data set of climate layers derived from monthly temperatures and rainfall recorded worldwide (Graham and Hijmans, 2006), were downloaded from WorldClim (www.worldclim.org). We used nineteen bioclimatic variables and altitude with 30 seconds (ca. 1 km) spatial resolution (Hijmans et al., 2005).

2.2.2. Model

MaxEnt 3.3.3k (Phillips et al., 2006) provides the option to use a range of functional forms to describe the relationship between presence data and environmental variables. These functional forms are known as feature class (Elith, et al., 2011). A combination of feature classes and regularization multiplier provided better results than the default settings

(Syfert et. al., 2013). According to Morales and colleges (2017) we adjusted the setting comparing different model combinations of the restriction feature class (lineal, quadratic, product, threshold, and hinge) and regularization multiplier (0.5, 1 and 1.5). We assessed 36 model combinations; I used 10 replicates for each model, this allows testing the model performance and created an average. I ran all model combinations and performed a Jackknife test (Peterson and Cohoon, 1999) , successively deleted variables with less than 2% contribution until all variables had a greater than 2 percent contribution, then selected the best model using the higher area under (the receiver operator characteristic) the curve (AUC). AUC values of 0.5–0.7 indicate low accuracy, values of 0.7–0.9 indicate useful applications and high accuracy. The output format selected to create distribution range map was the mean of the cumulative format, because it provides estimates of the suitable condition by environmental variables (best conditions at 100, unsuitable near 0) and using maximum training sensitivity plus specificity. The response curves were used to show how each environmental variable affects the MaxEnt prediction. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value. Finally, we used ArcGIS 10.2.2® for visualizing the result.

2.2.3. Historical temperature and precipitation

I analyzed the most contributed bioclimatic variables against the historical data of precipitation and temperature from Adjuntas as a representative location of the coffee growing region in Puerto Rico. Historical data was obtained from The Southeast Regional Climate Center (https://sercc.com/) and consists in a monthly average from 1970 to 2012.

Additionally, we included a representation of stages of the coffee tree phenology for Puerto Rico (Mariño et al., 2016).

2.2.4. Relationship with Percent of infestation

To estimate the percent of infestation by CBB in each farm site, we randomly collected one branch at breast height from nine plants. The percent of infestation was calculated as total CBB-bored berry divided by total of berries in each branch.

ArcGIS 10.2.2® software was used to superimpose the CBB occurrence data upon the MaxEnt suitability outputs and the most influential bioclimatic variable map to extract the values for each occurrence point. To evaluate the relation between coffee bored fruit percentage and suitability index I performed a linear model using R (R Development Core Team, 2019).

2.3. Result

The major setting combination was quadratic, product, hinge with 0.5 of regularization multiplier. This model performed better than random, AUC values for the mean of 10 replications (0.913 ± 0.02) for the given set of training, this suggests that the model was highly accurate for distinguishing between suitable and unsuitable areas for coffee berry borer (Figure 1).

According to the continuous average maps for CBB, the model projected highly suitable areas (80-100 %) for all its current known localities, also, it was suggested other potential suitable areas in the east of the island in which coffee was grown in the past (Figure 2).

The three most contributive variables to create the potential distribution model, according to the Jackknife test were, precipitation of wettest quarter (30.56%), altitude (22.21%), and precipitation seasonality (18.33%) (Table 2).

According to the marginal response curves for the variables that contributed strongly to the model building and the suitability for CBB grew exponentially with the precipitation of wettest quarter and elevation (Figure 3 A-B). The highest suitability for CBB presence was indicated in areas with the precipitation of wettest quarter ranged between 1000 – 1050 mm (Figure 3A). The optimum elevation range was between 400 to 1400 meters above sea level (masl) (Figure 3B). The CBB suitability was low in areas with low precipitation seasonality (<35 %), then increased exponentially reaching a peak (45-50 %), then slightly decreased with the increasing of the precipitation seasonality (Figure 3C). Precipitation of driest quarter under 200 mm predicted low suitability, then increased exponentially until it reaches peak between 250 to 300 mm (Figure 3D). Isothermality predicted high suitability until 73.5 % (Figure 3E). Furthermore, higher suitability was observed with Precipitation of Wettest Month which reached 350 mm, then decreased quickly (Figure 3F). Higher suitability was predicted between 10.0 to 10.5 °C mean diurnal range (Figure 3G). Finally, for temperature annual range under 13.0 °C predicted low suitability, then increased exponentially until it reached peak between 13.5 to 14.0°C (Figure 3H).

From historical climate data (1970-2012), the precipitation of wettest quarter (August to October) the average was around 300 mm and coincided with the coffee harvest period. Precipitation of driest quarter (January to March) was less than 100 mm defining most of the inter-harvest period. Highest temperature (±23 °C) from May to September corresponding with fruit growth stage. Moreover, the lowest (±19.5 °C) were between

December to February encompassing post-harvest and inter-harvest periods (Figure 4). I found a weak positive linear relationship (p-value= 0.036, R^2=0.16) between the suitability for CBB presence index and infestation levels (Figure 5).

2.4. Discussion

This study maps suitable habitat for CBB in Puerto Rico. The most suitable habitat for CBB, predicted for our model, overlaps the current known coffee growing areas in the island, which are in the central-west region of the Central Mountain Range (Flores, 2011). The entire, current coffee growing area is suitable for CBB, however, it shows different degrees that could vary year to year according the variability of bioclimatic variables and pest control practices. In congruence with several already published studies, my results showed the high ability of MaxEnt to produce prediction distribution models for the insets pest (Crawford and Hoagland, 2010; Ning et al., 2017).

Two of the most important variables for the suitability in the CBB presence predicted by the model are associated with precipitation, which affects the dynamics of infestation of the fruits by CBB, promoting the emergence, search and infestation of new fruits (Baker et al., 1992b; Constantino et al., 2011). The highest levels of infestation have been reported when relative humidity is elevated between 90 and 100% and decreases when it is less than 80% (Baker et al., 1992a) and the CBB development and survival were improved between 90-95% of relative humidity (Baker et. al., 1994).

The variable with higher contribution to the model was precipitation of wettest quarter, which coincides with the harvest stage and it is when the berries are in optimal condition for the development of CBB (Camilo et al., 2003). Altitude is among other factor, which appeared to limit the distribution of the CBB, this variable is related with humidity and

temperature (Austin, 2002) and is also associated with the highest CBB infection levels (Baker et al., 1989; Jonsson et al., 2015), and more difficult CBB control (Westly, 2010). For Puerto Rico, a significant and positive relationship has been reported between altitude and CBB infestation (Mariño et. al., 2017). Our presence data ranged from 55 to 966 masl, including the lowest and some of the highest coffee farms in the island. In summary, the weather climate conditions are determining the phenological development of the plant, influences pest developmental synchronization to the plant.

The precipitation of driest quarter based on historical data match with inter-harvest stage, this favors survival of CBB in fruits that remained in a branch and fall to the soil during and after harvesting, this is one of the main sources of infestation for the next harvest (Bustillo et al., 1998), allowing greater outbreaks of the CBB on dry periods than during rainy seasons (Constantino et al., 2010). On the other hand, the prolonged dry conditions could reduce CBB populations by berry desiccation (Baker, 1999). Climate change scenarios for the Caribbean indicate that the rainy season may become wetter and the dry season drier (Solomon et al., 2007), Harmsen et al., (2009) confirmed this pattern for Adjuntas, one of the most important coffee growth municipality in Puerto Rico.

Isothermality, temperature mean diurnal range are associated with the reduction in the life cycle of CBBin laboratory conditions (Jaramillo et al., 2009). On the other hand, temperature annual range are related with increase in the number of generations and number of female eggs per year (Jaramillo et al., 2010).

The model showed a weak positive relation between CBB infestation levels and MaxEnt suitable index, these results agreed with Bradley (2016) which concludes that models based on occurrence data are poor predictors of species abundance, but, location-based

models with high abundance can effectively predict regional patterns of abundance. Additionally, our presence data were from farms having different intensities of management, coffee plants growing in direct sunlight, under total or partial shade and even abandoned coffee areas, such may be affecting infestation levels and total population per fruit (Jonsson et al. 2015; Mariño et al., 2016; Sánchez et al., 2013).

2.5. Conclusion

In conclusion, this study provides important information about highly suitable habitat and main climatic variables which drives CBB distribution in Puerto Rico. These results can be used as a reference for understanding the impact of climate change on CBB and the influence of environmental factors on the development, reproduction, and survival of the insect; furthermore, for developing management strategies, generate early warnings regarding climate change and variability so that coffee growers can take timely control measures at specific locations, times or critical periods of infestation. The main bioclimatic variables identified on this study should also be further analyzed in detail in order to better understand the peculiarity of their influences in the pest annual predictability and on the development of better pest control strategies.

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2.7. Chapter II Tables

Chapter II **Table 1.** The environmental variables and altitude used to predict the suitable of Coffee berry borer habitat.

Variable	Description
Altitude	Elevation (meters above sea level, masl)
BIO1 =	Annual mean temperature (^{O}C)
BIO2 =	Mean diurnal range (mean of monthly (max temp - min temp)) (^{O}C)
BIO3 =	Isothermality (BIO2/BIO7) (*100)
BIO4 =	Temperature seasonality (standard deviation * 100)
BIO5 =	Max temperature of warmest month (^{O}C)
BIO6 =	Min temperature of coldest month (^{O}C)
BIO7 =	Temperature annual range (BIO5-BIO6) (^{O}C)
BIO8 =	Mean temperature of wettest quarter
BIO9 =	Mean temperature of driest quarter (^{O}C)
BIO10 =	Mean temperature of warmest quarter (^{O}C)
BIO11 =	Mean temperature of coldest quarter (^{O}C)
BIO12 =	Annual precipitation (mm)
BIO13 =	Precipitation of wettest month (mm)
BIO14 =	Precipitation of Driest Month (mm)
BIO15 =	Precipitation Seasonality (Coefficient of Variation)
BIO16 =	Precipitation of Wettest Quarter (mm)
BIO17 =	Precipitation of Driest Quarter (mm)
BIO18 =	Precipitation of Warmest Quarter (mm)
BIO19 =	Precipitation of Coldest Quarter (mm)

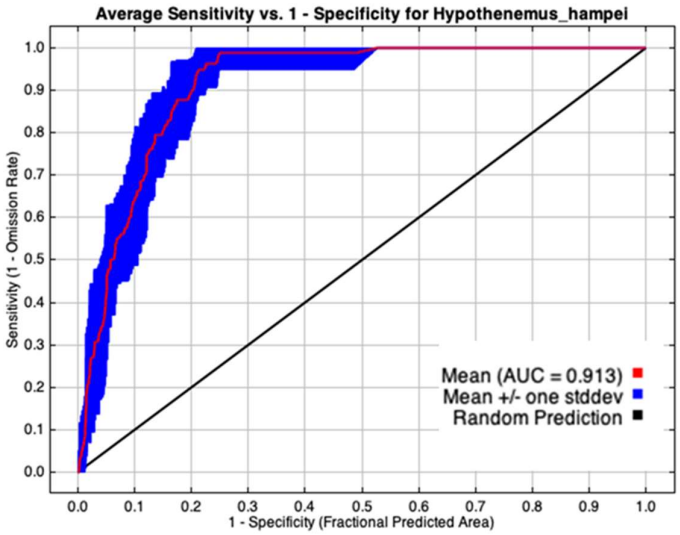
Seasonality is calculated by the standard deviation (temperature, in °C * 10) or coefficient of variation (precipitation in mm).

Chapter II **Table 2.** Relative contributions of the environmental variables to the MaxEnt model in predicting the suitable of Coffee berry borer habitat; values were averaged across 10 replicate runs.

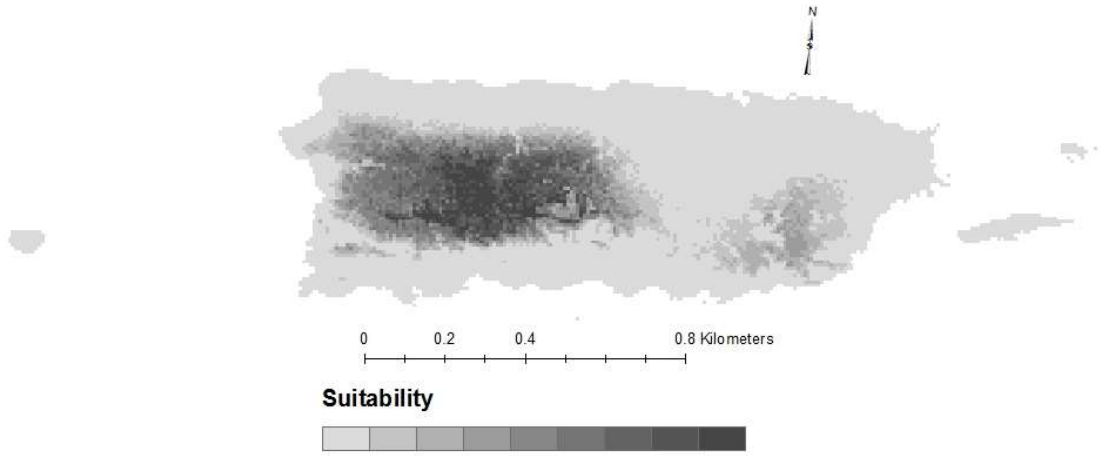
Variable	Percent contribution	Mean	SD	Min	Max
Precipitation of Wettest Quarter (mm)	30.56	904.60	116.37	658	1086
Elevation (masl)	22.21	547.35	215.16	82	995
Precipitation Seasonality (CV)[+]	18.33	43.81	3.59	36	52
Precipitation of Driest Quarter (mm)	7.27	255.78	23.87	146	327
Isothermality (BIO2/BIO7) (*100)	6.86	74.54	1.35	72	78
Precipitation of Wettest Month (mm)	5.61	320.44	41.42	240	391
Mean Diurnal Range (^{O}C)[++]	5.34	10.39	0.46	9.6	11.7
Temperature Annual Range (BIO5-BIO6)	3.82	13.84	0.39	13.2	15.0

[+] Coefficient of Variation in %. [++] (Mean of monthly (max temp - min temp)). General statistics were calculated using all occurrences (n = 162). (Min = minimum, Max = maximum, and SD = standard deviation).

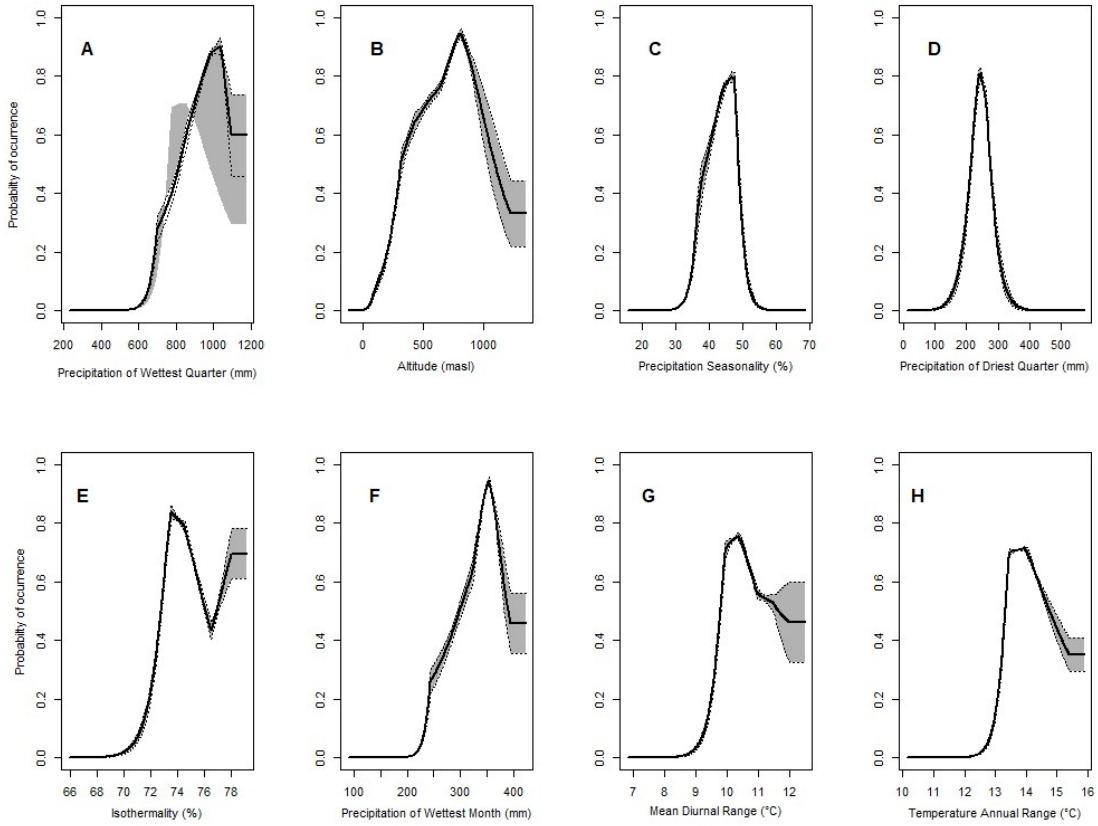
2.8. Chapter II Figures



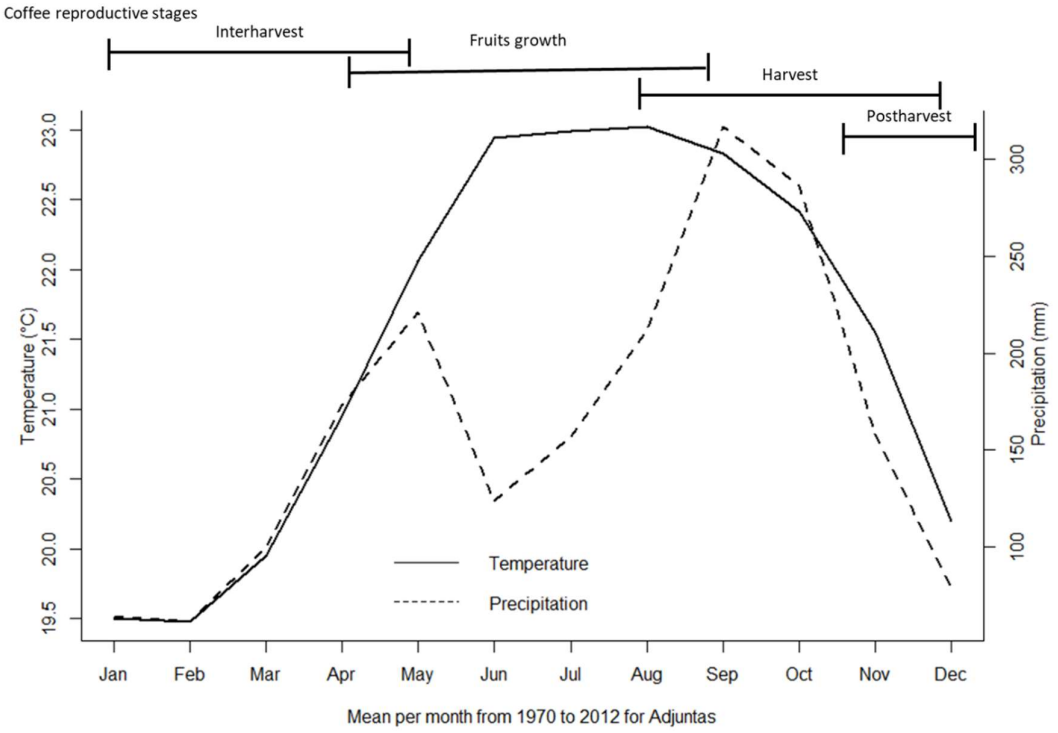
Chapter II. **Figure 1.**The Area Under a Receiver Operating Characteristic (ROC) curve (AUC) is summarizing the ability of the continuous diagnostic sampling to discriminate between suitable and non-suitable habitat. The current model indicated (AUC = 0.913) that can distinguish better than random model with 91.3% of chance between suitable and non-suitable habitat for *Hypothenemus hampei* in Puerto Rico.



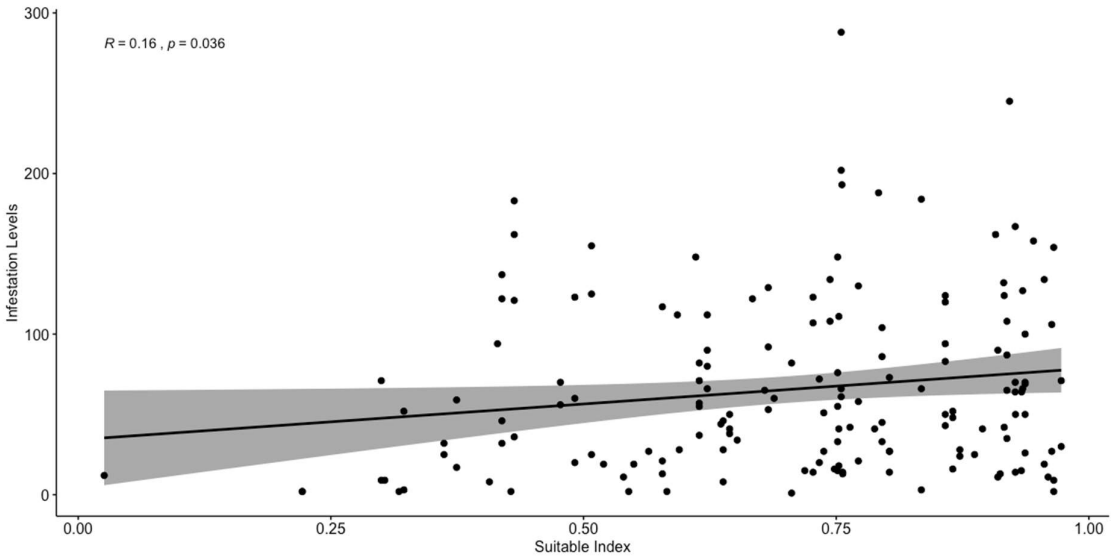
Chapter II. **Figure 2.** Model for suitable habitat for *Hypothenemus hampei* in Puerto Rico using MaxEnt. Darker colors are representing areas with better predicted suitability conditions. Darker areas are more suitable for the insect.



Chapter II. **Figure 3.** Marginal response curves of the predicted probability of *Hypothenemus hampei* occurrence for seven bioclimatic variables and altitude. showing how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value. The curves show the mean response of the 10 replicate MaxEnt runs (black curve) and the mean +/- one standard deviation (grey area).



Chapter II. **Figure 4.**Historical temperature (°C) and precipitation (mm) for Adjuntas, Puerto Rico from 1970 to 2012. Coffee reproductive stages in Puerto Rico are shown on the above lines.



Chapter II. **Figure 5.**The relation between *Hypothenemus hampei* infestation levels (CBB-bored berries/total of berries) in Puerto Rico and generated MaxEnt suitable index.

CHAPTER III

Isolation and identification of entomopathogenic nematodes in Puerto Rico coffee growth zone.

Abstract

Entomopathogenic nematodes (EPNs) are an available biocontrol possibility for insects with cryptic habit. The origin of the agents plays an important role in biological control efficacy since they could be more or less well-adapted to the biotic and abiotic challenges. The aims of this study were to isolate and identify natives EPNs present in the coffee soils. Soil samples were collected from 77 different sites located within 32 coffee farms distributed in the main coffee production area of the island. Nematodes were extracted from the soil using insect bait method with last instar of *Galleria mellonella*. Morphometric and molecular analysis were used to identify the isolates. Nematodes were recovered from 76 of the 95 soil samples (80%). Two potential EPN species were identified based on morphometrical and molecular traits, *Oscheius myriophila* (139 identified, 90.85%) and *Rhabditis rainai* (4 identified, 2.61%). No difference was found for morphometric comparison between juveniles of *O. myriophila* from different locations (pvalor> 0.05). To our knowledge this is the first record of *O. myriophila* isolation and colony establishment from a Puerto Rico isolate. This survey suggests that *O. myriophila* is a common and wide-spread EPN in the mountainous part of the island. Due to its prevalence and its interaction with other species, this species represents a high potential target for the development of biological control programs that considers EPNs.

3.1. Introduction

Entomopathogenic nematodes (EPNs) are a group of nematodes that inhabit the soil and are obligate parasites of insects (Grewal et al., 2005). Live symbiotically with pathogenic bacteria in order to kill the host insect and then feed the tissues of the insect cadaver and proliferating bacterial cells (Shapiro-Ilan et al., 2002). EPNs are widely recognized as a potent inundative biological control agent against a variety of insect pests (Grewal et al., 2005) by the higher potential to diminish harmful pests which cause economic, health and environmental damages (Gaugler et al, 2002).

Entomopathogenic nematodes have been successfully identified and used commercially in agricultural industries, forestry and medical entomology to control soil dwelling insects for decades (Campos-Herrera, 2015a). Have been described EPN in 23 families (Koppenhöfer, 2007) and the genera *Heterorhabditis* and *Steinernema*, include the largest number of species (Stock, 2015). Currently, more than 100 species of *Steinernema* and 16 species of *Heterorhabditis* have been described and continuously research is conducted to increase the possibility of identifying more species (Shapiro-Ilan et al. 2017). Recently, other members of the Rhabditida in *Oscheius* genus have shown potential to infect insects and are promising as new candidates for biocontrol of insect pests (Liu et al., 2012).

Coffee is the fifth most important crop in Puerto Rico, especially in the west-central mountainous region (Flores, 2011). Insects pests are one of the main constrained factors affecting the coffee yield and quality. More than 900 species of insects that feed on coffee in the world, however not all are considered of economic importance (Waller et al., 2007). Within these, coffee berry borer (CBB) *Hypothenemus hampei* Ferrari (1967), first time

reported In Puerto Rico was in 2007 (NAPPO, 2007), is considered the most destructive pest in all coffee-producing areas worldwide (Soto-Pinto et al., 2002). CBB infests the coffee beans and spends most of its life inside beans, remaining on mature and dry fruits (raisins) that be found in the soil between seasons, which makes it difficult to control (Baker et al., 1992). For cryptic habit pests, is highly recommend the use of biological control tools such as EPNs (Bustillo et al., 1998), especially for the population that survives for the next season shelter inside the fruits that fall to the soil during and after harvesting (Castaño et. al., 2005). Previous studies reported that *Steinernema feltiae*, *Heterorhabditis bacteiophora* and *Steinernema carpocapsae*, have the ability to move, penetrate the fruit resulting in high CBB mortality (Baker, 1999; Manton et. al., 2012; Molina and López, 2002; Molina et. al., 2009) once inside, NEPs can infect the different biological states of the CBB (Lara et. al. 2004). The EPNs have shown efficiency in the control of CBB under laboratory conditions (Castillo and Marbán-Mendoza, 1996; Manton et. al., 2012) as well as in the field (Manton et. al., 2012; Molina-Acevedo and López-Núñez, 2002; Molina-Acevedo and López-Núñez, 2009). On the other hand, *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) has been found parasitizing larvae, pupae and adults causing partial or complete sterilization of adult CBB females (Poinar et al., 2004).

For the biological control programs, indigenous organisms are usually preferred since they are more adapted to the biotic and abiotic threats of the ecosystem (Gaugler et al, 2002). The introduction of exotic EPNs may induce exclusion of the local nematode populations and be inefficient towards local insect pests as they may not be adapted to local environmental conditions (Miller and Barbercheck, 2001). Around 15% of the published research on biocontrol agents in Puerto Rico was dedicated to EPNs. (Gallardo-Covas, 2017). *Heterorhabditis* sp. was the first report ed EPN occurring in Puerto Rico (Roman and

Beavers, 1983). The identification of indigenous EPN populations in Puerto Rico will provide more options for nematode-based biological control, implementation with Integrated Pest Management (IPM) and management of invasive insect pests, such as *Hypothenemus hampei* (coffee berry borer, CBB) and others coffee pests. Therefore, the aims of this study were to survey, isolate, and identify natives EPNs present in the coffee growing areas.

3.2. Methodology

3.2.1. Soil samples

Soil samples were collected in a total of 32 coffee farms in the main coffee production area in Puerto Rico, which is in the central mountain range. It is a humid region with an average annual precipitation of 1905 mm and presents an altitude range between 35 - 982 meters above sea level. The farms were different agronomic management, diverse plantation age, coffee plants growing in direct sunlight and under total or partial shade. On each farm, three blocks (15m×15m) were sampled for a total of 95 blocks sampled. In each block, using a shovel was taken a soil sample of approximately 2 kg composed of five subsamples located under the coffee trees canopy, ranging from 0 to 15 cm of depth. The samples were placed in plastic bags (26.7 cm X 27.3 cm), closed to prevent humidity loss and transported in a cold container. Previously the use the tools were disinfected with a solution of hypochlorite 5% and rinsed with water.

3.2.2. Entomopathogenic nematode isolation

The soil samples were sifted with a sieve number 8 (opening 2.36 mm, U.S.A. standard sieve series) to remove segments of roots, stones and to homogenize. Between each sample, this sieve was washed with water, soap, 1% of sodium hypochlorite, heated in an

oven at 85^0C for 15 minutes and disinfected with 95% alcohol. For isolation of entomopathogenic nematodes from the soil was used the baiting technique (Bedding and Akhurst 1975). Three plastic containers of 500mL, having a perforated lid, were filled with representative soil for each replica and added 10 larvae of last instar of wax worm, *Galleria mellonella,* and incubated for 7 days at 29^0C in complete darkness. In order to facilitate the movement of larvae in the soil and the infection by EPN the soil was gently placed without compression in the plastic container. After the incubation period the larvae were carefully extracted and individually placed in White tramps (White, 1927) containing Ringer solution. The larvae were assessed for nematodes infection after 7 days. The nematodes that were found were reinoculated in *G. mellonela* larvae to confirm the pathogenicity and for further multiplication. Finally, isolate nematodes were store in 200mL tissue culture flask (Model) containing 100mL distilled water at 15^0C and complete darkness.

3.2.3. Morphometric identification

For morphometric analysis the isolates N281 (Adjuntas), N304 (Mayaguez), N322 (Maricao), N336 (Ciales) and N260 (Jayuya), IJ were selected. The in vivo reproduction of EPNs was performed using *Galleria mellonella* larvae because it was suitable to be used for the multiplication of most species of EPN (Dutky et al 1964). *Galleria mellonella* were inoculated and incubated in a Petri dish on a filter paper at 25 °C. Infective juveniles were collected after 7 days from the cadavers. Juveniles of each isolate were slide-mounted using a modified agar method (Grewal, 1990) containing 2 g of agar (Frontier Scientific Service, Cat. # 7060) dissolved in 100 ml of distillate water by heating for 1:30 min in a microwave. The agar was allowed to cool and whilst still warm, poured into a glass Petri dish. After the agar solidifies a small quantity of the agar was taken from the Petri dish and

place on the slide. The nematodes were killed in a 4 % formaldehyde for approximately 1 hour. Using a micropipette under stereoscopic (40x Magnification) nematodes transferred in a drop to the small piece of agar. Finally, the cover glass was carefully placed on the top of the drop. Measurements were made under the Leica DM2500 light microscope of the main morphological characteristics. Photo and measurement were taken with the Leica DMC 2900 camera and with the Leica Application Suite software (LASver.4). Morphological observations were total body length (L), maximum body width (W), distance from anterior end to base excretory pore (EP), distance from anterior end to nerve ring (NR), distance from anterior end base of basal bulb (ES), tail length (T), ABW: anal body width, a: Total body length divided by maximum body width (L/W), b: Total body length divided by distance from anterior end base of basal bulb (L/ES), c: Total body length divided by tail length (L/T) following the taxonomic criteria by Hominick et al. (1997).

3.2.4. Molecular identification of EPN

3.2.4.1. DNA extraction of EPN

Genomic DNA was obtained using five individuals for each nematode sample. It was performed using a protocol of QIAamp DNA Mini kit (Qiagen Inc, Cat. # 51306, Germany). Before the DNA extraction and PCR process, all pipets were cleaned with hypochlorite 10%. Additionally, the pipets, the tips and tubes were put under UV light for 20 minutes.
The sample was added to the tube (1.5mL), 50 µl of PBS buffer and 1 measure of a micro-spoon (100 µl) of zirconium beads 1mm (Next Advance, New York, USA), with. Then the samples were macerated in a Bullet blender (Next Advance Model BBY24MR, New York, USA) for 3 minutes at speed 12. Afterward added 180 µl of buffer ATL, 20 µl of proteinase K, and incubate in a hot water bath (56°C) for approximately 1 hour until the tissue

particles have dissolved. After that, the samples, were centrifuged for 30 seconds at 13200rpm, added 4 µl of RNaseA (100mg/ml), then vortex and incubated at room temperature for 2 minutes. We centrifuged the samples again at 13200 rpm for 30 seconds and added 200 µl of AL buffer, vortex and incubated in a hot water bath at 70°C for 10 minutes. Then, we added 200 µl of Ethanol molecular grade, vortex and centrifuged for 30 seconds at 13200rpm. The samples were put in the QIAamp mini spin column and centrifuged at 8000 rpm for 1 minute. We then changed the used collection tube of the column for a new one and discarded the liquid on it. We added 500 µl of AW1 buffer, centrifuged for 1 min at 8000 rpm, and changed again the collection tube for a new one and discarded the liquid. We then added 500 µl of AW2 buffer, centrifuge 3 minutes at 13200 rpm, and change the collection tube and centrifuge again for 1 minute at 13200rpm. We finally put the column in a new clean 1.5 ml Eppendorf tube, discarded the used collection tube and eluted the DNA with 50 µl of AE Elution buffer and incubation at room temperature for 1 hour. We centrifuged the samples for1 min at 8000 rpm, and again for 1 min at 13200rpm. A NanoDrop spectrophotometer (Thermo Scientific Inc. NanoDrop 2000) was used to evaluate the concentration and quality of DNA obtained. In order to confirm the presence and quality of the extracted DNA, 5µl was used for electrophoresis (30minutes at 90 volts) in 1X TBE buffer on 1% agarose gel, stained with loading dye (2µl), visualized and photographed under UV light.

A polymerase chain reaction (PCR) was done to amplify the internal transcribed spacer (ITS) region. This region is highly conserved in most nematode species and can be used to identify newly isolated nematode species. A PCR amplification of nematodes ITS rDNA region was made using the universal primers D2A(5'-CAAGTACCGTGAGGGAAAGTTG-3')

and D3B (5'TCGGAAGGAACCAGCTACTA-3'), to amplify the D2A/D3B expansion segment of 28S rDNA (Al-Banna et al. 1997). The DNA was standardized to 3-6 ng/µl. The PCR conditions followed protocol by Mráček et. al., (2006) with the optimized annealing temperature of 57 °C. Using a thermocycler (Model 2720 Thermal Cycler-Applied Biosystems), PCR was run with a final volume of 25 µL per reaction with the following temperature conditions: 94 °C for 7 minutes; followed by 35 cycles of 94 °C for 60 seconds, 57 °C for 60 seconds, 72 °C for 60 seconds; and a final extension of 72 °C for 10 minutes. Then, electrophoresis was performed in 1% agarose gel, 75 volts, 40 minutes, to confirm the amplification of the expected DNA fragment.

The PCR product was cleaned with the kit QIAquick PCR purification Kit (Qiagen Inc, Cat. # 28106, Germany). Final electrophoresis was performed in 1% agarose gel, 75 volts, 40 minutes, to confirm that the DNA amplicon was cleaned and with enough concentration for sequencing. Finally, a nanodrop measured the cleaned DNA amplicons to determine the concentration.

Sequencing of the amplicons was performed at Macrogen (Korea) using the same primers from the original PCR reaction. A reagent mix containing 2 µ L Big Dye, 3.2 mmol sense primers, 3.0 µ L of the amplified product containing 400 ng DNA and 2.0 mL of water was prepared for the product end of the PCR reaction. The reaction for sequencing was carried out according to manufacturer's instructions Applied Biosystems 3730XL sequencers). The sequences of the different nematode isolates were edited using the CodonCode Aligner v6.0.2 (CodonCode Corporation, Dedham, MA, USA) to correct any base-calling error. Then, these sequences were aligned by using MEGA-X v10.1 (Kumar, et al., 2018). Once aligned, a

BLAST (Basic Local Alignment Search Tool) was made (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the species with the highest percentage of similarity. The sequences were deposited into the GenBank database. Finally, maximum likelihood trees were reconstructed using RAxML v2.2.3 (Stamatakis, 2006) on CIPRES with 25 discrete gamma categories. Bootstrap analysis was automatically determined. *Heterorhabditis bacteriophora* was used as the out-group.

3.2.5. Statistical analysis

An EPN positive block was considered when at least one of the 10 larvae showed the presence of nematodes. Detection frequency (DF) was calculated as a positive block divided by the total block. Shapiro-Wilk test was conducted assess the normality of the data. One way of Analysis of Variance (ANOVA) was performed on the ten morphometric variables for juvenile of five selected *Oscheius myriophila* isolates. Statistical analysis was conducted in R (R Core Team, 2019).

3.3. Results

Soil samples were collected from 95 blocks (15x15m) from 32 coffee farms (Figure 1). *Galleria mellonella* larvae, used as bait tramps, containing nematodes were retrieved from 80% of the 95 blocks (76 were positive for EPNs). The detection frequency by block range 0 to 46.67 % for a total of 361 nematode colonies isolated. We identified 153 of 361 (42.38%) isolated nematodes, 132 of which (93.46 %) were considered as potential EPNs. The potential EPNs identified were *Oscheius myriophila* (139 identified, 90.85%) and *Rhabditis rainai* (4 identified, 2.61%). A total of 128 new sequences were archived in GenBank under accession numbers MN389602 – MN389729 (Table A.1).

The maximum likelihood phylogenetic tree (Figure 2) based on rDNA 28S D2/D3 sequences of isolates EPNs, revealed that the isolate *O. myriophila* grouped with AY602176 *O. myriophila* with a 95% and *Rhabditis rainai* with EU195966 *R. rainai* with a 98% bootstrap support.

The measurements of morphometric traits (Table 1) is presented for the juveniles of *Oscheius myriophila* (Figure 1B) isolates N281 (MN389691), N304 (MN389702), N322 (MN389714), N336 (MN389726) and N260 (MN389676) (Figure 1A). No statistical differences were observed among the ten morphometric variables ($p \leq 0.05$). Total body length (L) F=2.062, p= 0.093 (W = 0.99108, p-value = 0.7967), maximum body width(W) F= 1.274, p= 0.286 (W = 0.98787, p-value = 0.5596), distance from anterior end to base excretory pore (EP) F=1.843, p= 0.128 (W = 0.99191, p-value = 0.8517), distance from anterior end to nerve ring (NR) F= 0.4 p= 0.750 (W = 0.99448, p-value = 0.9706), distance from anterior end base of basal bulb (ES) F= 0.394, p = 0.812 (W = 0.97591, p-value = 0.08616), tail length (T) F= 2.398, p= 0.056 (W = 0.98706 , p-value = 0.5027), anal body width (ABW) F= 0.313, p= 0.868 (W = 0.95454, p-value = 0.2826), a:Total body length divided by maximum body width (L/W) F= 1.239 p= 0.300 (W = 0.98899, p-value = 0.6423), b: Total body length divided by distance from anterior end base of basal bulb (L/ES) F= 0.571, p= 0.650 (W = 0.96456, p-value = 0.1329), c: Total body length divided by tail length (L/T) F= 2.447, p= 0.052 (W = 0.99089, p-value = 0.7827).

3.4. Discussion

The present study aimed at determining the natural occurrence of EPN in the Puerto Rico coffee growing area, representing the most systematic and extensive effort made in the island to evaluate indigenous species of EPNs. The survey covered representative

bioclimatic regions in the main coffee growing area. The results suggest that EPNs were well distributed in the survey area at the time of sampling (from September 2015 to April 2017)). The recovery frequency (80%) could be attributed to the agroecosystem of coffee farms in Puerto Rico which exhibits less disturbed soils and low amounts of inorganic fertilizer and pesticides that are applied. These conditions were reported to be associated with a high prevalence of EPNs (Shapiro et al., 1999). A similar result was observed in Kenya when comparing several agroecosystems (forest, pasture, coffee and vegetable garden) reporting highest detection in coffee (Niyasani et. al., 2008).

Most of the nematode isolates reported here belong to the genus *Oscheius* Andrássy, 1976, which were isolated from 18 (90.85%) out of 20 positive soil samples. Overall the phylogenetic trees confirm the current isolates to be within the genus *Oscheius* and clustered within the Insectivora group. This genus is easy and commonly isolated from soil samples (Félix et al., 2001). *Oscheius* is part of the family Rhabditidae Örley, 1880. Actually, few studies demonstrating *Oscheius* contribution to biological control, since most species of this genus might have a facultative-parasite habit (Ye et al., 2010). The first entomopathogenic genus identified in the family Rhabditidae was *Heterorhabditidoides*, then later was suggested as synonym of *Oscheius*, and proposed that the name of the type species of *Heterorhabditidoides* should be changed to *Oscheius* (Liu et al., 2012).

The genus *Oscheius* can be characterized as insect-parasitic (Godfrey et al., 2005). This is composed of two main groups, *Dolichura* and *Insectivorus* (Sudhaus and Hooper 1994). The *insectivorus* group represents various associations with invertebrate hosts ranging from facultative to obligate parasitism (Liu et al., 2012). Although, originally has not been described as an EPN, nowadays it is recognized as a nematode that parasitizes insects and that has great potential as a biological control agent (Dillman, 2012). The main

characteristics to consider a nematode as an entomopathogen are: the presence of a mutualistic-symbiotic relationship with pathogenic bacteria, the relationship between might be facultative, although it is maintained over subsequent generations and the death of the insect is less than 5 days (Dillman et al., 2012). Under these criteria *O. chongmingensis, O. carolinensis, O. gingeri and O. safricana* were recently reported as true entomopathogens able to penetrate their insect host through the spiracles, colonize, develop completely to the adult in the host and kill insects tested in the laboratory (Zhang et al., 2008; Torres-Barragan et al., 2011; Pervez et al., 2013; Serepa-Dlamini and Gray, 2018). They also were found to be mutually associated with parasitic and lethal to some insect pests Gram-negative bacteria belonging to the *Enterobacteriaceae* family, genus *Serratia* (Liu et al., 2012; Torres-Barragan et al., 2011; Ye et al., 2010) and showing similarities with the association that steinernematids and heterorhabditis with its respectively symbiotic bacteria (Lephoto et al. 2015; Serepa-Dlamini and Gray, 2014; Torrini et al. 2015).

Initially, only in the *Insectivorus* group were found EPNs (Pervez et al. 2013). But nevertheless, *O. onirici, O. tipulae* and *O. karachiensis* on the *Dolichura* group have been reported capable of infecting larvae of *Galleria mellonella* and *Tenebrio molitor* (Torrini et al., 2015; Karimi et al., 2018; Mehmood and Khanum, 2018). Nevertheless, the species in the *Dolichura* group needs further studies for confirmation the entomopathogenicity (Campos- Herrera et al. 2015b).

The most frequently identified nematode in this study was *Oscheius myriophila*. Belongs to the *insectivora* group (Tabassum at al., 2016) and phylogenetic analysis showed that *O. myriophila* is closely related to *O. chongmingensis O. colombianus* and *O. insectivorus*

suggesting it as a possible entomopathogenic nematode (Zhang et al., 2008; Al-Zaidawi et al., 2019). Poinar (1986) stated that the full pathogenicity of *O. myriophila* was still not clear. In fact, not all isolated *O. myriophila* were able to reinfect and reproduce successfully in *Galleria mellonella* larvae. According to Schulte and Sudhaus (1989) is possible, that *O. myriophila* like others *Oscheius*, can be a facultative entomopathogenic nematode when the nematode carries some pathogenic bacteria, they kill the host. On the contrary, when the nematode carries only a harmless bacterium, they wait for the host die, as a necromenic (wait for the host die) organisms. Despite this, Dillman (2012) classified *O. myriophila* as potential EPN and consider necromenic behavior as an intermediate evolutionary stage between entomopathogenic and parasitism.

Another potential entomopathogenic nematode isolated was *Rhabditis rainai*, and its relationship with the insects was described as phoretic, moderately pathogenic, and facultatively parasitic (Osbrink and Carta, 2005).

Morphological traits resembled the original description of *Oscheius* (Poinar, 1986). Other described isolates suggested differences in having larger body length and distance from the head to the nerve ring, and smaller tail length and width at anus with original description (Erbaş et. al., 2017). Lacey and Georgis (2012) propose that geographical origin and habitat can influence nematode morphology, but nevertheless, we found, the non-significative difference in morphometry trait between isolates from different regions on the island. *Oscheius* can be dispersed by human movement of plants, soils, agricultural products (Pimentel et al. 2005) and phoretic behavior (Eng et al. 2005). On the other hand, we found wide range of detection frequency in each block sampled. This agreed with studies carried

out by Campos-Herrera and Gutierrez (2014) who reported that nematodes isolated in different locations, from the same species, can differ in mortality, the time to kill the larva, and the penetration percentage.

3.5. Conclusion and recommendation

The present study is the first tentative of a comprehensive survey that indicates the presence, incidence and distribution of potential entomopathogenic activity of nematodes isolated from soils in the Puerto Rico coffee growth region. This is the first documented record of *Oscheius myriophila* isolation from the island, its broad occurrence and prevalence in the surveyed areas indicating its potential role in the natural regulation of insect populations and as a biocontrol agent. Certainly, the identification of new tentative endemic entomopathogenic nematodes is a welcomed addition to the list of biocontrol agents. However, we recommend testing against other insects, field studies and identification of pathogenic symbiotic bacteria and further biotypes characterization. Therefore, increasing our knowledge about species diversity of insect parasitic nematodes provides vast opportunities for conducting research, and develop new tools that could be used in sustainable agriculture systems.

3.6. References

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3.7. Chapter III Table

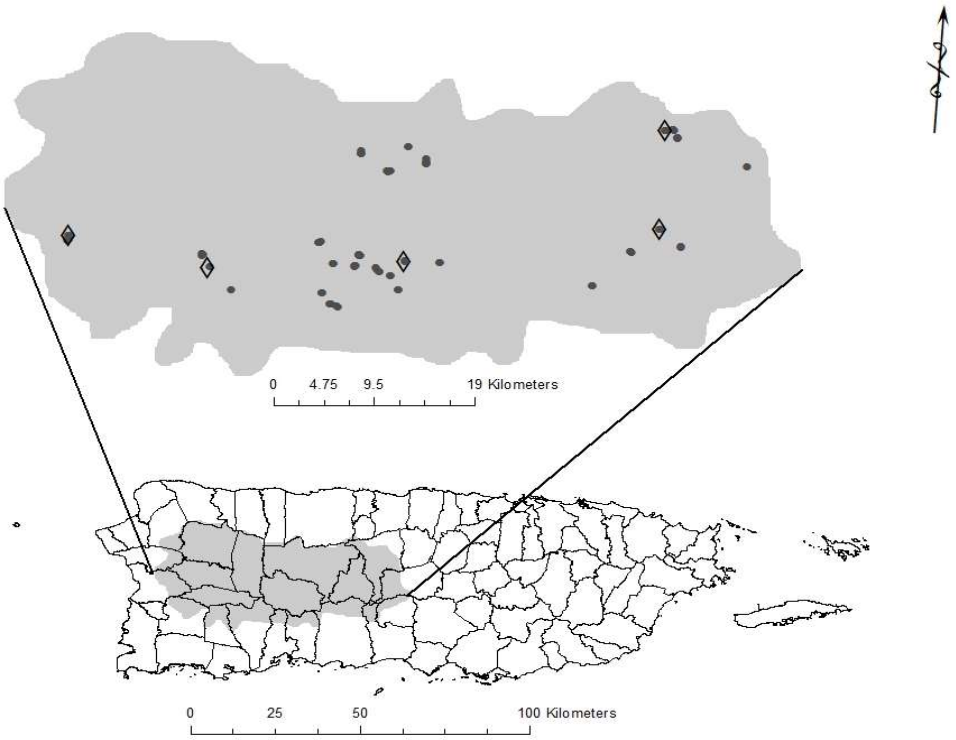
Chapter III. **Table 1**. Morphometrics data of five infective juveniles isolates of *Oscheius myriophila*. All measurements are in µm and in the form: mean±SD (range).

Sample	N281 (MN389691)	N304 (MN389702)	N322 (MN389714)	N336 (MN389726)	N260 (MN389676)	*Oscheius myriophila
Location	Adjuntas 18.17983 N -66.77331 W	Mayaguez 18.20403 N -67.05818 W	Maricao 18.17365 N -66.94017 W	Ciales 18.3036 N -66.5521 W	Jayuya 18.21028 N -66.55675 W	
n	22	20	17	19	14	6
L	569.42±1.49 (567-573)	569.49±2.30 (566-573)	571.79±2.34 (567-575)	569.14±2.21 (565-573)	569.06±1.19 (567-572)	564 (504-611)
W	24.9±1.4 (22.4-27.6)	25.5±2.42 (20.2-29.3)	24.2±1.57 (21.4-26.9)	24.5±2.17 (21.8-29.1)	25.1±1.60 (22.4-27.7)	23 (19-26)
EP	104.99±2.03 (100.69-100.68)	104.34±1.82 (100.68-107.50)	105.56±2.23 (100.27-100.28)	106.03±2.92 (102.02-110.77)	105.81±1.60 (110.77-107.93)	107 (97-114)
NR	89.19±2.76 (83.77-93.48)	88.78±2.06 (85.10-94.19)	88.73± (83.22-92.96)	89.56±2.29 (87.07-91.88)	88.90±1.80 (86.26-92.73)	89 (83-96)
ES	130.94±1.94 (125.59-134.30)	131.06±2.24 (125.40-134.59)	130.86±1.80 (127.46-133.95)	130.69±1.88 (125.71-133.27)	131.54±2.17 (128.50-134.97)	129 (126-136)
T	80.14±1.72 (77.04-83.37)	80.10±1.55 (76.56-83.02)	80.01±1.56 (76.90-83.13)	81.33±2.05 (78.13-85.96)	79.47±2.31 (76.07-84.08)	78 (75-80)
ABW	13.71±1.99 (7.99-17.17)	14.36±3.04 (9.26-21.96)	13.69±1.78 (9.75-17.47)	13.93±2.65 (8.27-19.01)	14.39±3.33 (9.21-21.35)	15 (14-16)
a	22.94±1.28 (20.77-25.35)	22.58±2.30 (19.38-28.27)	23.69±1.57 (21.34-26.70)	23.38±1.94 (19.47-25.90)	22.72±1.44 (20.58-25.47)	N/A
b	4.35±0.06 (4.24-4.53)	4.34±0.07 (4.24-4.56)	4.36±0.07 (4.25-4.50)	4.35±0.06 (4.26-4.54)	4.33±0.07 (4.21-4.44)	N/A
c	7.11±0.16 (6.81-7.39)	7.12±0.14 (6.90-7.43)	7.14±0.14 (6.92-7.37)	7.00±0.19 (6.57-7.32)	7.12±0.21 (6.77-7.49)	N/A

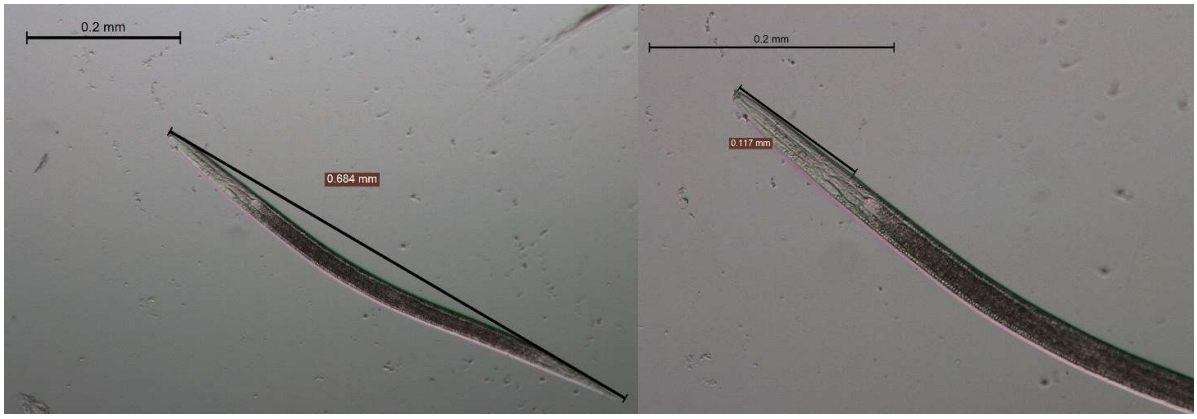
L: total body length, W: maximum body width, EP: distance from anterior end to base excretory pore, NR: distance from anterior end to nerve ring, ES: distance from anterior end base of basal bulb, T: tail length, ABW: anal body width, a: L/W, b: L/ES, c: L/T. *Holotype description *Oscheius (= Rhabditis) myriophila* (Poinar, 1986)

3.8. Chapter III Figures

A



B



Chapter III. **Figure 1.** A) Map of Puerto Rico showing the distribution of sites sampled for nematodes. The ◊ represent the location of sampled used to morphometric analysis. The gray area represents the main coffee-growing area of Puerto Rico. B) Infective juvenile of *Oscheius myriophila*

A


EU195965_1_Rhabditis_blumi
98
N248(MN389669)
99
N252 (MN389672)
EU195966_1_Rhabditis_rainai
EU195996_1_Oscheius_guentheri
100
HM355819_1_Oscheius_dolichura
100
EU195971_1_Oscheius_dolichura
100
HM355818_1_Oscheius_dolichura
83
MF196098_1_Oscheius_tipulae
EU195969_1_Oscheius_tipulae
89
FJ547239_1_Oscheius_carolinensis
EU195968_1_Oscheius_insectivora
N170(MN389650)
N196(MN389662)
N272(MN389686)
N180 (MN389656)
N253(MN389673)
N269 (MN389683)
N334(MN389725)
100
N324(MN389716)
N299 (MN389699)
N319 (MN389711)
N300 (MN389700)
N84 (MN389609)
N259 (MN389675)
N92(MN389612)
N332(MN389723)
N274(MN389687)
N271(MN389685)
N246 (MN389668)
N261(MN389677)
N333(MN389724)
N279 (MN389690)
N284 (MN389693)
N320 (MN389712)
N314 (MN389707)
N305(MN389703)
N91(MN389611)
42
N270 (MN389684)
N168 (MN389648)
N275 (MN389688)
N301(MN389701)
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N136(MN389632)
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N244 (MN389667)
N78 (MN389608)
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N110 (MN389619)
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N288 (MN389694)
98
N199 (MN389664)
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N123(MN389627)
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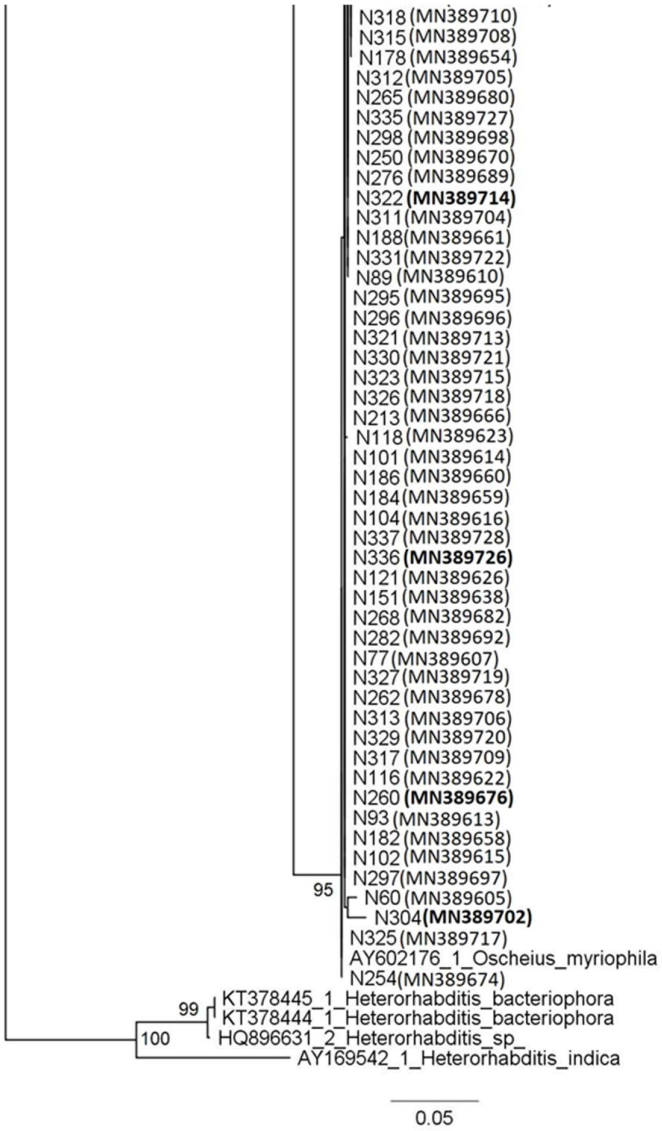
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N297 (MN389697)
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AY169542_1_Heterorhabditis_indica
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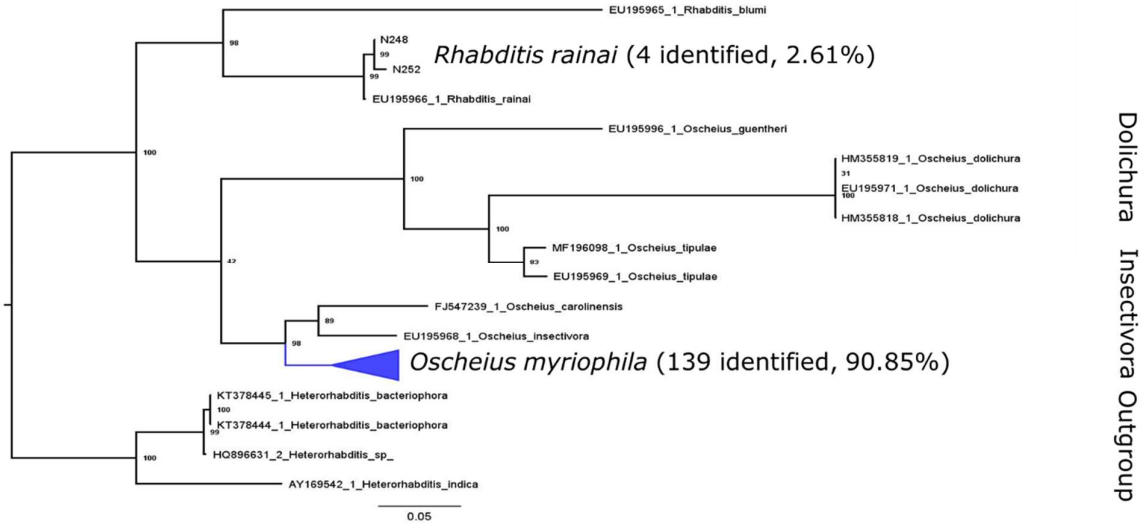
B



Chapter III. **Figure 2.** (A) Maximum likelihood phylogenetic tree showing the relationship between *Oscheius myriophila* isolates and their similarity with those from the GenBank based on 631 bp expansion D2/D3 sequences 28S rDNA region. *Heterorhabditis bacteriophora* was used as an outgroup. In bold isolates used to morphometric analysis. In bold isolates used in morphometric analysis. (B) Summary of the Figure 2A were the *O. myriophila* populations were compressed.

3.9. Chapter III Appendix

Table A 1. Accession number and location of isolates entomopathogenic nematodes

Lab ID	Accession number	Collection date	Country	Organism	Lat_Lon
N16	MN389602	10/9/2015	Yauco	*Oscheius myriophila*	18,15054 N -66,84315 W
N22	MN389603	10/23/2015	Lares	*Oscheius myriophila*	18,19839 N -66,84441 W
N36	MN389604	4/27/2016	Adjuntas	*Oscheius myriophila*	18,15293 N -66,77886 W
N60	MN389605	5/20/2016	Utuado	*Oscheius myriophila*	18,27721 N -66,75477 W
N64	MN389606	5/20/2016	Utuado	*Oscheius myriophila*	18,27721 N -66,75477 W
N77	MN389607	5/20/2016	Utuado	*Oscheius myriophila*	18,27237 N -66,75505 W
N78	MN389608	5/20/2016	Utuado	*Oscheius myriophila*	18,27237 N -66,75505 W
N84	MN389609	5/20/2016	Utuado	*Oscheius myriophila*	18,27325 N -66,75504 W
N89	MN389610	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N91	MN389611	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N92	MN389612	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N93	MN389613	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N101	MN389614	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N102	MN389615	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N104	MN389616	5/20/2016	Utuado	*Oscheius myriophila*	18,27366 N -66,75519 W
N107	MN389617	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18652 N -66,81202 W
N108	MN389618	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18652 N -66,81202 W
N110	MN389619	6/2/2016	Adjuntas	*Oscheius myriophila*	18,18652 N -66,81202 W
N111	MN389620	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18652 N -66,81202 W
N115	MN389621	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18652 N -66,81202 W
N116	MN389622	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N118	MN389623	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N119	MN389624	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N120	MN389625	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N121	MN389626	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N123	MN389627	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N125	MN389628	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N129	MN389629	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18631 N -66,81214 W
N132	MN389630	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18601 N -66,81184 W
N135	MN389631	6/3/2016	Adjuntas	*Oscheius myriophila*	18,18601 N -66,81184 W
N136	MN389632	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N137	MN389633	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N139	MN389634	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N142	MN389635	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N143	MN389636	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N144	MN389637	6/3/2016	Adjuntas	*Oscheius myriophila*	18,1784 N -66,74322 W
N151	MN389638	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17846 N -66,74284 W
N155	MN389639	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N156	MN389640	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W

N157	MN389641	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N158	MN389642	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N161	MN389643	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N162	MN389644	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N163	MN389645	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N164	MN389646	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N167	MN389647	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N168	MN389648	6/3/2016	Adjuntas	*Oscheius myriophila*	18,17912 N -66,74335 W
N169	MN389649	6/17/2016	Ponce	*Oscheius myriophila*	18,1564 N -66,6137 W
N170	MN389650	6/17/2016	Ponce	*Oscheius myriophila*	18,1564 N -66,6137 W
N174	MN389651	6/17/2016	Ponce	*Oscheius myriophila*	18,1566 N -66,6136 W
N176	MN389652	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N177	MN389653	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N178	MN389654	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N179	MN389655	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N180	MN389656	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N181	MN389657	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N182	MN389658	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N184	MN389659	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N186	MN389660	6/17/2016	Ponce	*Oscheius myriophila*	18,1568 N -66,6138 W
N188	MN389661	7/10/2016	Lares	*Oscheius myriophila*	18,17777 N -66,83392 W
N196	MN389662	7/16/2017	Adjuntas	*Oscheius myriophila*	18,16613 N -66,78528 W
N197	MN389663	7/16/2017	Adjuntas	*Oscheius myriophila*	18,16613 N -66,78528 W
N199	MN389664	7/16/2017	Adjuntas	*Oscheius myriophila*	18,16613 N -66,78528 W
N200	MN389665	7/16/2017	Adjuntas	*Oscheius myriophila*	18,16613 N -66,78528 W
N213	MN389666	7/16/2017	Adjuntas	*Oscheius myriophila*	18,16642 N -66,78507 W
N244	MN389667	8/3/2017	Jayuya	*Oscheius myriophila*	18,19342 N -66,53831 W
N246	MN389668	8/3/2017	Jayuya	*Oscheius myriophila*	18,21009 N -66,55666 W
N248	MN389669	8/3/2017	Jayuya	*Rhabditis rainai*	18,21009 N -66,55666 W
N250	MN389670	8/3/2017	Jayuya	*Oscheius myriophila*	18,21009 N -66,55666 W
N251	MN389671	8/3/2017	Jayuya	*Oscheius myriophila*	18,21043 N -66,55663 W
N252	MN389672	8/3/2017	Jayuya	*Rhabditis rainai*	18,21043 N -66,55663 W
N253	MN389673	8/3/2017	Jayuya	*Oscheius myriophila*	18,21043 N -66,55663 W
N254	MN389674	8/3/2017	Jayuya	*Oscheius myriophila*	18,21043 N -66,55663 W
N259	MN389675	8/3/2017	Jayuya	*Oscheius myriophila*	18,21028 N -66,55675 W
N260	MN389676	8/3/2017	Jayuya	*Oscheius myriophila*	18,21028 N -66,55675 W
N261	MN389677	8/3/2017	Jayuya	*Oscheius myriophila*	18,21028 N -66,55675 W
N262	MN389678	8/8/2017	Adjuntas	*Oscheius myriophila*	18,18547 N -66,81141 W
N263	MN389679	8/5/2017	Adjuntas	*Oscheius myriophila*	18,18547 N -66,81141 W
N265	MN389680	8/5/2017	Adjuntas	*Oscheius myriophila*	18,18564 N -66,8113 W
N266	MN389681	8/5/2017	Adjuntas	*Oscheius myriophila*	18,18564 N -66,8113 W
N268	MN389682	8/5/2017	Adjuntas	*Oscheius myriophila*	18,17019 N -66,79432 W
N269	MN389683	8/5/2017	Adjuntas	*Oscheius myriophila*	18,17019 N -66,79432 W
N270	MN389684	8/5/2017	Adjuntas	*Oscheius myriophila*	18,17032 N -66,79484 W
N271	MN389685	8/5/2017	Adjuntas	*Oscheius myriophila*	18,17032 N -66,79484 W

N272	MN389686	8/5/2017	Adjuntas	*Oscheius myriophila*	18,17032 N -66,79484 W
N274	MN389687	8/5/2017	Adjuntas	*Oscheius myriophila*	18,18036 N -66,77295 W
N275	MN389688	8/8/2017	Adjuntas	*Oscheius myriophila*	18,18036 N -66,77295 W
N276	MN389689	8/8/2017	Adjuntas	*Oscheius myriophila*	18,18036 N -66,77295 W
N279	MN389690	8/8/2017	Adjuntas	*Oscheius myriophila*	18,18036 N -66,77295 W
N281	MN389691	8/8/2017	Adjuntas	*Oscheius myriophila*	18,17983 N -66,77331 W
N282	MN389692	8/8/2017	Adjuntas	*Oscheius myriophila*	18,17968 N -66,77314 W
N284	MN389693	8/8/2017	Adjuntas	*Oscheius myriophila*	18,17968 N -66,77314 W
N288	MN389694	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20082 N -67,05871 W
N295	MN389695	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N296	MN389696	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N297	MN389697	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N298	MN389698	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N299	MN389699	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N300	MN389700	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20392 N -67,05826 W
N301	MN389701	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20403 N -67,05818 W
N304	MN389702	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20403 N -67,05818 W
N305	MN389703	7/21/2017	Mayaguez	*Oscheius myriophila*	18,20403 N -67,05818 W
N311	MN389704	11/29/2017	Maricao	*Oscheius myriophila*	18,15253 N -66,92081 W
N312	MN389705	11/29/2017	Maricao	*Oscheius myriophila*	18,15253 N -66,92081 W
N313	MN389706	11/29/2017	Maricao	*Oscheius myriophila*	18,15253 N -66,92081 W
N314	MN389707	11/29/2017	Maricao	*Oscheius myriophila*	18,17467 N -66,93846 W
N315	MN389708	11/29/2017	Maricao	*Oscheius myriophila*	18,17467 N -66,93846 W
N317	MN389709	11/29/2017	Maricao	*Oscheius myriophila*	18,17467 N -66,93846 W
N318	MN389710	11/29/2017	Maricao	*Oscheius myriophila*	18,17467 N -66,93846 W
N319	MN389711	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N320	MN389712	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N321	MN389713	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N322	MN389714	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N323	MN389715	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N324	MN389716	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N325	MN389717	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N326	MN389718	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N327	MN389719	11/29/2017	Maricao	*Oscheius myriophila*	18,17365 N -66,94017 W
N329	MN389720	11/29/2017	Maricao	*Oscheius myriophila*	18,1857 N -66,94505 W
N330	MN389721	11/29/2017	Maricao	*Oscheius myriophila*	18,1857 N -66,94505 W
N331	MN389722	11/29/2017	Maricao	*Oscheius myriophila*	18,18613 N -66,94473 W
N332	MN389723	11/29/2017	Maricao	*Oscheius myriophila*	18,18613 N -66,94473 W
N333	MN389724	11/29/2017	Maricao	*Oscheius myriophila*	18,18706 N -66,94559 W
N334	MN389725	11/29/2017	Maricao	*Oscheius myriophila*	18,18706 N -66,94559 W
N335	MN389726	12/1/2017	Ciales	*Oscheius myriophila*	18,3036 N -66,5521 W
N336	MN389727	12/1/2017	Ciales	*Oscheius myriophila*	18,3036 N -66,5521 W
N337	MN389728	12/1/2017	Ciales	*Oscheius myriophila*	18,3036 N -66,5521 W
N338	MN389729	12/1/2017	Ciales	*Oscheius myriophila*	18,3036 N -66,5521 W

Chapter IV

Occurrence of *Oscheius myriophila* (Rhabditidae: Rhabditida) in Puerto Rico Coffee growing area.

Abstract

A survey for native entomopathogenic nematodes in Puerto Rico Coffee growing area was conducted. The most frequent species isolated was *Oscheius myriophila*, considered as a potential biocontrol agent. The survival, distribution and proper use as biocontrol agents depend on abiotic factors, such as soil and its physicochemical properties and environmental conditions. The aim of this research is to assess the influence of some abiotic variables on the occurrence of *O. myriophila*. Ninety-three soil samples were collected through the main coffee producer area and entomopathogenic nematodes were trapped using the insect bait method with *Galleria mellonella*. Each sample replica was characterized by agroecosystem (sun or shade coffee growth) and physicochemical soil properties. *Oscheius myriophila* was detected in shade (84.21%) and sun (73.68%) areas. Likewise, it was found in a variety of soil texture classes, such as, clay, clay loam, loam, sandy clay loam, sandy loam, silty clay and silty clay loam. The presence of *O. myriophila* was positively correlated with P and F concentrations. A logistic regression model showed a strong relationship between *O. myrophila* frequency and coffee agroecosystems, soil texture classification, pH and altitude. No significative relation was found with percentage of organic matter, accounting for 84% of the total variation. By understanding the variables that influence the natural occurrence of *O. myriophila* it is possible to enhance its further performance as part of a biocontrol program.

Keywords: Agroecosystem, entomopathogenic nematodes (EPNs), *Oscheius myriophila.*

4.1. Introduction

Coffee is the second most traded commodity in the world, its production has now spread to 70 countries where (ICC 2009). Approximately 7.5 million tons were consumed each year, therefore it is considered one of the world's most popular beverages (Killeen and Harper, 2016). In Puerto Rico, coffee is grown in mountainous regions (Muñiz and Monroig, 1994) and is one of the most important crops (Flores, 2011).

Entomopathogenic nematodes (EPN) have already been effectively utilized to manage the numerous insect pests of agricultural importance (Shapiro-Ilan et al., 2006), especially against soil inhabiting insect pests having cryptic behavior (Wright, 1992). Most of the commercial EPN species used for biological control programs belong to the families Steinernematidae and Heterorhabditidae (Gaugler et al., 2002). Recently, nematodes belong to the genus *Oscheius* were reported as EPNs (Torres-Barragan et al., 2011). EPNs penetrate into a host, release their symbiotic bacteria from the intestine into the host's hemolymph where they propagate and kill the host by septicemia in 48 h (Hominick, 2002). NEPs have special attributes as biocontrol agents, especially, their wide range of potential hosts, possibility of massive production and overall environmental safety (Grewal 2012). Soil is the natural environment of entomopathogenic nematodes (Kaya, 2002). Therefore, survival, distribution and efficacy as biocontrol agents depend on factors such as ecosystem type, soil texture (the size range of particles), soil pH and altitude (Lawrence et al.,2006; Yoshida et al., 1998). Also, soil fertility regulating the nematofauna (Mulder and Vonk, 2011) and the environmental availability of key nutrients, are very important ecological constraints influencing species abundance (Elser, 2006).

Ecosystem type has a strong influence on nematode accumulations (Zhong et al., 2016). Coffee agroecosystems are interactive networks consisting of anthropogenic, meteorological, edaphic and biological components (Wagenet, 1998). Based on production systems is possible to distinguish two main coffee agroecosystems prevailing in Puerto Rico: First, most traditional coffee growing

practices involve growing coffee under a diverse canopy of native or introduced forest trees in high to moderate shade (Mariño et al., 2016). Trees protects the coffee plants from harsh wind, excessive light and soil erosion, and they buffer temperature and humidity. Furthermore, shaded plantations support biodiversity comparable to that in some rain forests (Moguel and Toledo 1999). However, shade significantly decreases the availability of photosynthetic radiation negatively affecting the yield (Morais et al. 2003). Second, most modern and intensive practice consists in grown coffee in full sun to increase net yields. Sun growing plantations typically experience greater soil run-off, nutrient leaching, low biodiversity and usually high inputs of agrochemicals (Perfecto et al., 1996). The differences between sun and shade coffee agroecosystems may influence the effectiveness and adoption of biological control practices (Gámez-Virués et al., 2012: Karp et al., 2013). The effects of plant species on the nematodes depends on the ecosystem and type of plant (Alumai et al., 2006).

The EPN species *Oscheius myriophila* belongs to the insectivora group of *Oscheius* genus (Tabassum at al., 2016). Despite the fact that its pathogenicity is still not fully clear (Poinar 1986), this is considered as potential entomopathogenic nematode (Dillman, 2012). The influence of agroecosystem, soil physical and chemical characteristics on the occurrence of *O. myriophila* still remains poorly known. In essays at our laboratory, *O. myriophila* showed to be able to infect both larva and adult of CBB (Figure 1B, Unpublished results).

The understanding of the natural occurrence of *O. myriophila* populations offers insights about the optimal conditions for suitable incorporation as a biocontrol agent in the coffee crops; about chemical characteristics of soil, agroecosystems and habitat preference (sun or shade coffee), how soil type (texture classification, organic content), pH, and altitude are affecting their occurrence. The main aim of this work was to assess the influence of some abiotic variables on the occurrence of *O. myriophila* in Puerto Rico main mountain coffee growing area.

4.2. Methodology

4.2.1. Soil samples

Soil samples were collected from 24 coffee farms in the main coffee production area in Puerto Rico (Figure 1). Each farm was subdivided into 3 block site replicates (15m×15m) for a total of 75 sites. In each replicate, a soil sample was collected of approximately 2 kg composed of five subsamples. The subsamples were collected under the coffee trees canopy, ranging from 0 to 15 cm in soil depth. For a shade coffee, the samples were collected under the canopy of coffee trees under the canopy of shade trees.

4.2.2. Nematodes

The *Oscheius myriophila* isolates found in this study were collected using a standard *Galleria mellonella* baiting technique (Bedding and Akhurst, 1975) in an extensive EPNs survey conducted in Puerto Rico mountain region. Each soil sample was distributed among three plastic containers, one pound of capacity each. Then, 10 larvae of *G. mellonella* (the last instar) were placed in the plastic container (30 larvae total) and incubated for 7 days at 29^0C in complete darkness. After 7 days, dead insects were carefully removed and placed in modified White traps (White, 1929) and were assessed if infested with nematodes after 7 days. For all sample it was calculated the Detection Frequency (DF) based on the occurrence of larva infected with nematodes and divided by total larvae per replica.

4.2.3. Physical and chemical characteristics of soil

Soil samples were submitted to the soil nutrient analysis laboratory at the University of Connecticut (Storrs, CT 06269) for the Standard Nutrient Analysis to determine available soil nutrient such as calcium, magnesium, phosphorus, potassium, sulfur, iron, manganese, copper, zinc, aluminum and boron using a modified Morgan extractant method. Soluble salt measure electrical conductivity and the soil pH were also provided. The percent organic matter in soil determined by the loss on ignition and the total amounts of sand, silt and clay was determined using a hydrometer method.

Soils were categorized according to USDA soil texture classifications (USDA 1987). Moreover, the field sample replicas were identified based on altitude, coffee growing type as a sun (direct sunlight) and shade (under total or partial shade).

4.2.4. Statistical analysis

The relationship between reported occurrence of *O. myriophila* and soil fertility properties was analyzed through Spearman correlation. Moreover, a logistic regression model with Poisson distribution was used to describe the probability of nematode populations occurring given the particular set of coffee grow type (sun or shade), soil textures, soil pH and altitude of the site. Statistical analysis was conducted in R (R Core Team, 2019).

4.3. Results

The percentage of detection of *O. myriophila* with Galleria baits in seventy-three different coffee sites was 84.21 % shade growing type (total of 42) and 73.68 % sun coffee (total of 31). Soil samples harboring nematodes were found in soils with organic matter ranged from 2.00 to 10.9 % and soil pH from 4 to 6.4 % and altitude between of 228 to 975 masl. The detection frequency in different soil texture classes were clay (43.83%), clay loam (27.40%), loam (13.70%), sandy clay loam (5.48%), sandy loam (4.11%), silty clay (2.74%) and silty clay loam (2.74%). The occurrence of *O. myriophila* was significant positive associated with concentrations of Fe and P. In the same way, no significative correlation was observed between K, Ca, Mg, Al, B, Cu, Mn and Zn content and the percentage of occurrence of *O. myriophila* (Table 1).

Ocheius myrophila detection frequency was influenced significatively by the coffee agroecosystems, (p-valor <0.001) decreasing 25 % going from shadow to sun, soil texture classification (p-valor <0.001), decrease 35 % when the pH increases pH (p-valor <0.001), decrease -0.062 % when the altitude increases altitude (p-valor <0.004) and non-significative with organic matter (p-valor 0.284), accounting for 84% of the total variation (Table 2). Figure 2 illustrates the change in the probability of encountering *O. myrophila* associated to variation in five soil

characteristics or location significant explanatory variables. According to the model prediction, the probability detection of *O. myrophila* highest in shade coffee than sun coffee growth (Figure 2 A). For the soil texture, the model predicted a positive relation with silty clay loam and sandy clay loam classes. Furthermore, the model predicts a negative relation between the percentage of nematode detection and clay loam, silty clay loam, sandy loam classes. Any statistical relation with loam class was observed (Figure 2 B). The model also predicts a strong negative relationship between pH, altitude and the probability of trapping *O. myrophila* (Figure2 C and D).

4.4. Discussion

Since, nematodes belonging to the genus *Osceius* has been as a potential as a biological control agent (Dillman, 2012), several studies reported its pathogenicity again several insect pests (Zhang et al., 2008; Torres-Barragan et al., 2011; Pervez et al., 2013; Serepa-Dlamini and Gray, 2018). However, this is the first study highlighting the occurrence of *O. myriophila* according to defined abiotic factors such as soil fertility and intrinsically location characteristics of sampling sites.

Our results show a positive correlation between *O. myriophila* recovery using *Galleria* baits and Fe and P concentrations in soils. Iron content have been reported having a weak vitalizing effect on the infectivity of the nematodes with respect to *Galleria mellonella* (Campos-Herrera et. al 2008). Nevertheless, the effects of P on the nematodes still unclear, Alumai et al., (2006) suggest EPNs were associated with habitats with phosphorus and on the contrary, canonical correspondence analysis revealed that the presence and abundance of EPNs were inversely related to P content (Hoy et al., 2008).

In general, complex (shade coffee) landscapes are expected a higher abundance and diversity of natural enemies and more successful biocontrol than simplest (sun coffee) landscapes (Rusch et al., 2017). We found a relationship between *O. myriphila* detection and coffee agroecosystem type. The model predicted, as previous hypothesized, a higher detection probability in shade coffee than

in sun coffee. Shade have impact on soil temperature and moisture which are related with EPNs population density (Lawrence et al., 2006),

In our study, the soil pH was acidic, ranging from 4 to 6.4, and this is considered favorable for the survival of EPNs (Nyasani et al. 2008). For *O. myriophila* detection, our model predicted a negative relationship with % pH. Very acidic and highly alkaline soils significantly reduced the vitality of infective juvenile (Kung et al. 1990b). According to Rosa et al. (2000), pH 6 is a borderline for entomopathogenic nematode occurrence. However, they have been detected in a wide range of pH soil levels (Stock et al. 1999).

Altitude had a clear influence on the distribution of *O. myriophila* in the island. The greater frequency of occurrence of *O. myriophila* in areas situated from 228 to 975 masl, consistently with lower annual median temperature and higher rainfall, seems to suggest that these climatic conditions are more favorable to nematode survival. A similar pattern was observed for *Heterorhabditis* which was most abundant from lower altitudes, and its relative abundance decreased with altitude. Similarly, in Cameroon, nematodes belonging to the genders *Steinernema* and *Heterorhabditis* were encountered, in humid forest, more frequently at low elevation (Kanga et al., 2012).

Soil texture has been considered important variable affecting EPN populations, consequently, pore spaces and size are related to the size of the intermolecular spaces (for EPNs movement), the aeration and water availability (Kaya 1990). In general, recovering of nematodes is highly dependent on soil type (Duncan et al., 2013). This factor affects the hydrology and chemistry of soils, having major effects on the behavior of EPNs (Kaspi et al., 2010). The results of our study a suggest significative effect of soil textural classes on the detection probability of *O. myriphila*. According to Raheel et al. (2015), entomopathogenic nematodes are more frequently found in sandy loam soils. Our result suggested higher *O. myriphila* detection probability in soil with good potential porosity (sandy and silty). Previous study showed that EPNs were associated with habitats

with relatively high sand, and moderate silt (Alumai et al., 2006). This soil type provided good aeration and soil moisture retention, which are considered basic requirements for nematode survival in the soil (Kung, 1990a). Conversely, lower detection probability was found in soil with no poor potential porosity (clay and loam). Koppenhöfer and Fuzy (2006) reported that as clay content increases, activity of *Steinernema scarabaei*, *Steinernema glaseri*, *Heterorhabditis zealandica*, and *Heterorhabditis bacteriophora* decreases. These types of soils restrict nematode movement and are poorly aerated (Shapiro-Ilan et al. 2002). Evaluation of the efficacy of *Steinernema feltiae* (Rioja strain) against *Spodoptera littoralis* showed negative effects of clay content, suggesting that heavy soils reduce its virulence (Campos-Herrera and Gutiérrez, 2009).

Finally, in this study organic matter content (2.00 to 10.9 %) was not significantly related to the detection of *O. myriophila*. Apparently, the effect of organic matter on nematode infectivity varies among nematode species (Koppenhöfer and Fuzy, 2006). Thus, higher organic matter content increases persistence (Shapiro et al., 1999) and dispersion of EPNs (Wilson et al., 2012). Moreover, Kapranas et. al., (2017) suggest that the proportion of sand and organic matter content influence the foraging behavior of *Steinernema carpocapsae*, *Heterorhabditis downesi* and *Steinernema feltiae*.

4.5. Conclusion

The field and soil quantitative information of *O. myriophylla* generated from this study improve the understanding of habitat requirement, basic knowledge to optimize its use as a biocontrol agent in a particular soil type. The broad occurrence of *O. myriophila* in most of the surveyed areas indicates the potential role of these nematodes in the natural regulation of insect pest populations. The nematode *O. myriophila* is predominantly to be found in shade coffee, with relatively elevate sand content, low pH, and at lower coffee growing altitude.

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4.7. Chapter IV Tables

Chapter IV. **Table 1.** Association of soil fertility characteristics with the reported occurrence of *Oscheius myriophila* in main coffee growing area in Puerto Rico.

	Detection	K	Ca	P	Mg	Al	B	Cu	Fe	Mn	Zn
Detection											
K	0.03										
Ca	-0.24	0.53***									
P	**0.39****	0.1	-0.16								
Mg	-0.22	0.53***	0.85***	-0.17							
Al	0.14	-0.28	-0.68***	0.17	-0.56***						
B	-0.24	0.42*	0.58***	-0.04	0.45*	-0.56***					
Cu	0.06	0.15	-0.01	0.22	0.14	0.2	-0.06				
Fe	**0.28 ***	-0.23	-0.57***	0.38	-0.47**	0.79***	-0.49**	0.2			
Mn	-0.17	0.1	0.17	-0.49**	0.31	-0.26	0.02	0.13	-0.25		
Zn	0.11	0.24	0.26	0.51***	0.24	-0.31	0.17	0.31	-0.06	-0.02	
S	-0.23	0.12	0.01	-0.42*	-0.05	0.22	0.11	0.1	0.02	0.43*	-0.32

Level of significance Spearman's correlation, *P < 0.05; **P < 0.01, ***.

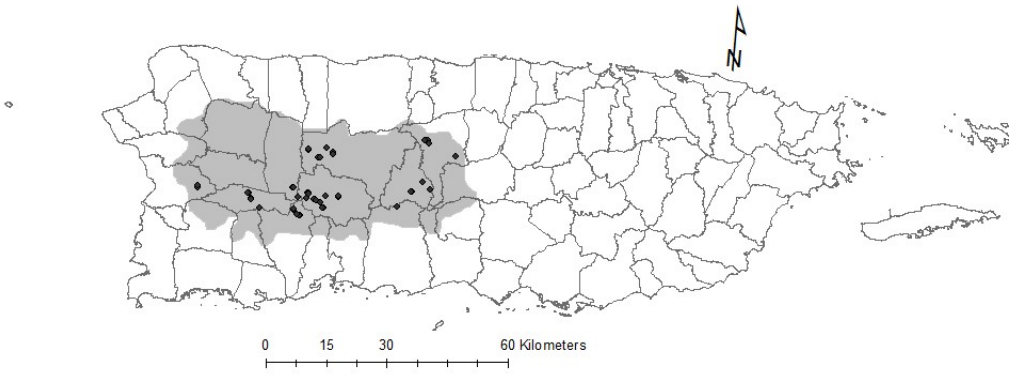
Chapter IV. **Table 2.** Logistic regression model applied the data of reported occurrence of *Ocheius myrophila* in Puerto Rico.

Coefficient	Incidence Rate Ratio	Estimado	Std. Error	Conf. Int (95%)	Statistic	P-Value*
Intercept	162.27	509	0.29	92.61 – 284.33	17.78	<0.001
Coffee agroecosystems	0.78	-25	0.07	0.68 – 0.90	-3.51	<0.001
Clay loam$^{\delta}$	0.82	-19	0.07	0.71 – 0.95	-2.59	0.009
Silty clay	0.69	-37	0.12	0.55 – 0.87	-3.15	0.002
Silty clay loam	1.47	39	0.11	1.19 – 1.82	3.56	<0.001
Loam	1.16	15	0.17	0.84 – 1.62	0.9	0.368
Sandy clay loam	1.41	35	0.17	1.02 – 1.96	2.08	0.038
Sandy loam	0.44	-82	0.32	0.23 – 0.83	-2.53	0.011
pH	0.7	-35	0.05	0.64 – 0.78	-6.89	<0.001
Altitude (masl)	1	-0.062	0	1.00 – 1.00	-2.88	0.004
Organic matter (%)	0.98	-2	0.02	0.94 – 1.02	-1.07	0.284

Observations 73, Nagelkerke's R20.859, $^{\delta}$ according to USDA soil texture classifications (1987), * p < 0,05 indicate significant categories.

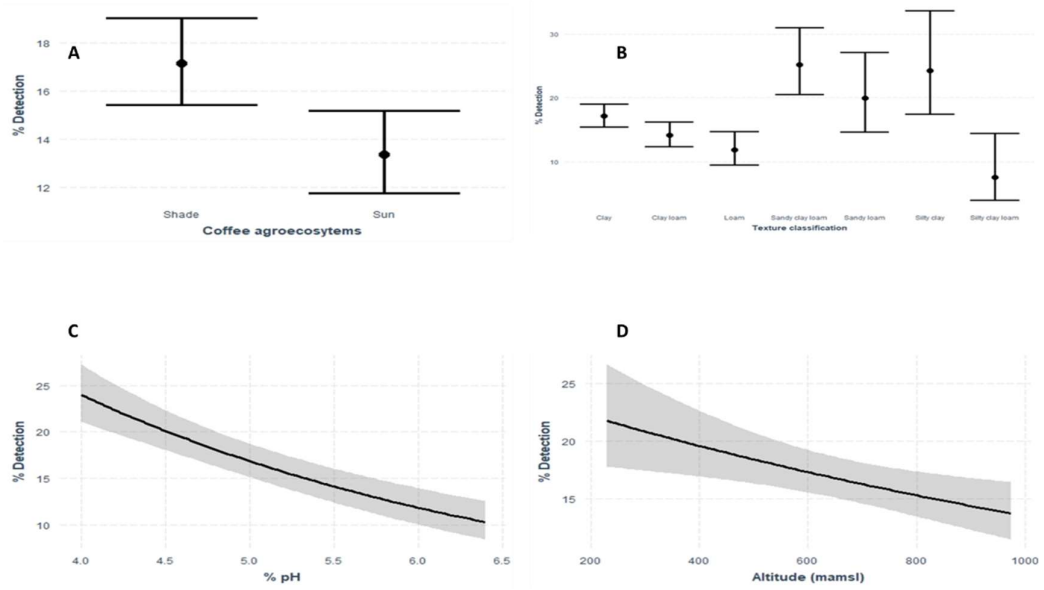
4.8. Chapter IV Figures

A



B

Chapter IV. **Figure 1.** A) Map of Puerto Rico showing distribution of the 24 soil sites in 72 farms, sampled for entomopathogen nematodes. The gray area represents the main coffee production zone in Puerto Rico. B) Coffee berry borer infected by *Oscheius myriophila* during experimental trial, San Juan, Puerto Rico (Bar = 0.5mm).



Chapter IV. **Figure 2.** Logistic regression model results for *Oscheius myrophila* showing the probability of encountering a single live individual plotted against each explanatory variable, (A) coffee agroecosystem, (B) texture classification, (C) pH and (D) altitude. In each graph, three variables were held constant with the fourth allowed to vary as plotted.

CHAPTER V

GENERA CONCLUSIONS AND RECOMMENDATIONS

The results of this dissertation are significant for developing coffee berry borer (CBB) integrated pest management programs involving *Oscheius myriophila* or other entomopathogenic nematodes (EPNs) as components. This study integrated spatial distribution of the suitable habitats for the prey (CBB), detection of potential biocontrol agents (EPNs) and influence of the factors on the natural occurrence and persistence of EPNs in any given site. This knowledge should allow us to apply EPNs more judiciously and to increase their efficacy against coffee pests.

We found broad suitability for the occurrence of CBB in all traditional coffee growing areas. This knowledge provides a reliable first baseline to understand the climatic factor which drives its distribution. Our methodology to validate the model results using historical climatic data and CBB field percent of infestation confirm that the use of MaxEnt, bioclimatic variables and elevation allow to effectively determine the distribution of the insets. Overall, our model result stablishes precipitation of wettest quarter, altitude and precipitation seasonality as a three most contributive variable to the distribution of CBB and also found a positive relationship between model suitable index and field infestation of CBB. We expect that understanding the effects of the climate on pest biology helps us to plan preventives management strategies.

We have extended the knowledge regarding EPNs in Puerto Rico. Our result showing a high prevalence of *Oscheius myriophila* throughout all coffee zone and make available relevant molecular and morphometric information. The findings of this potential native entomopathogenic nematodes is new and increasing our knowledge about insect pathogenic nematodes. This provides vast opportunities for conducting either fundamental or applied research, ultimately leading to new insights for biological control programs that

contribute to minimizing the use of chemical pesticide. Further studies of local EPNs are needed to assess the pathogenicity against insects of economic importance and to determine its effectiveness in the field.

Since nematodes belong to *Oscheius* genus have been recognizing as an EPNs, this the first study to assess the soil characteristics that influence its detection. The results of this study suggest that many factors interact to determine the occurrence of *O. myriophila*. My study enabled us to detect the positive effects of shade on the activity of *O. myriophila* and their abundance when compared with the full sun. These findings suggest that shade coffee may improve the action of this EPN. Furthermore, *O. myriophila* is likely to be found in sites with relatively high sand content, low pH and low altitude. Although the current analysis provides insight into the soil conditions associated with the nematodes at the time of the survey, the results of this study, show that he interaction of soil parameters such as pH, texture, and elevation coupled with shade coffee may help predict *O. myriophila* occurrence. We recommend assessing of several isolates of *O. myriophila* in each farm because different nematodes strain behaves differently in different soils.