Seasonality and Prevalence of the Fungal Trichomycete *Asellaria jatibonicua* in the Terrestrial Isopod *Litthorophiloscia culebrae*

by:

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RESUMEN

Los tricomicetos son microorganismos que viven mayormente en el intestino de artrópodos con mandíbula. Son considerados como un grupo ecológico compuesto de hongos (Asellariales y Harpellares) y protistas (Amoebidiales y Eccrinales). Para entender el comportamiento de estos simbiontes es necesario entender primero la biología de su hospedero, por lo cual comparamos la prevalencia de *Asellaria jatibonicua* en el intestino del isópodo terrestre *Litthorophiloscia culebrae* con diferentes parámetros ambientales tales como: porciento de humedad relativa, temperatura y precipitación de lluvia. Los isópodos fueron colectados semanalmente en la Universidad de Puerto Rico, Recinto de Mayagüez por un periodo de 18 meses. Se determinó una prevalencia promedio de *A. jatibonicua* de 26% con mayor prevalencia en los meses de mayor humedad relativa. Además, encontramos una nueva especie para Puerto Rico en el mismo hospedero, *Parataeniella* sp. (Ichthyosporea: Eccrinales), la cual describimos completamente.

ABSTRACT

The trichomycetes is a group of commensal microorganisms generally found in association with the digestive tract of mandibulated arthropods. They have been recognized as an ecological group composed of Kickxellomycotan fungi (Asellariales and Harpellales) and Ichthyosporean protists (Amoebidiales and Eccrinales). Isopods, in particular, harbor fungal members belonging to the Asellariales (rarely Harpellales). One essential aspect of the ecology of the Asellariales is to understand the environmental conditions that regulate isopod populations. For instance, the different environmental fluctuations of temperature, water precipitation and relative humidity affect isopod lifestyle and, thus potentially, the life cycle of these fungi. In this study, isopods were collected in the University of Puerto Rico - Mayagüez and dissected in the laboratory the same day, in order to verify the presence of *Asellaria jatibonicua*. We determined an average prevalence of 26% over 18 months and observed seasonality behavior of *Asellaria jatibonicua* associated with the terrestrial isopod *Litthorophiloscia culebrae* Moore, 1901. Data show a low prevalence of the fungus in the less humid months (with the lowest prevalence of 7% in August 2012 and 8% in March 2013), increasing towards the more humid months (with the highest prevalence of 44% in September 2012 and 55% in December 2012). In addition, during this study we found another trichomycete in the same host, *Parataeniella* sp. (Ichthyosporea: Eccrinales), which is described in this script and represents a first record for Puerto Rico and a new species for science.

Keywords: Terrestrial isopods, trichomycetes, Asellariales, Eccrinales, seasonality, prevalence.

DEDICATORY

To my parents James and Lisa, who have always loved me unconditionally and encouraged me to become a trustful person and a good professional. They never stopped believing in me and always told me that I was going to accomplish everything I wanted in my life and laid in the hands of God.

To my siblings Mariela, Karina, Ambar, Clara, and David, since life without them would not be the same.

To my friend, fiancé and love of my life, Antonio G. Rodríguez, who has been of great support emotionally in the moments of desperation over all these years and have never stopped encouraging me to become a better person.

Finally and most importantly, to God that gave me the strength every day to continue and completing my thesis' investigation and everything I have proposed in my heart. If it were not for Him and His awesome Creation I never have had the opportunity to study the wonderful and amazing details of biology.

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1. INTRODUCTION

Terrestrial isopods (Isopoda: Crustacea) play an important ecological role in organic matter decomposition, as members of the detritivore community. These arthropods represent a prominent component of the soil macrofauna, feeding on forest litter, rotten wood, fungi and bacteria (Sutton 1980). Detritivory is of great significance in forests, as the litter layer provides the major source of decomposable organic matter, which is essential for forest growth (Quadros and Araujo 2008). Terrestrial isopods participate mainly in the processing of litter, through mechanical breakdown and comminuting of the leaves. Since they generally have a low efficiency of assimilation, most of the ingested leaf litter returns to the soil as feces, which are chemically and physically different from the original vegetal material (Zimmer 2002). The feces are more suitable to microbial colonization and constitute a source of nutrient recycling for other soil organisms (David and Gillon 2002). As other arthropods, terrestrial isopods carry symbiotic microorganisms associated to their gut, which help the individuals to hydrolyze certain polymers such as cellulose, lignin and chitin (Wang et al. 2007). Moreover, isopods show commensalistic interactions with trichomycetes.

The trichomycetes are cosmopolitan obligate symbionts inhabiting the gut of arthropods, generally associated in a commensal relationship (Siri and López Lastra 2010). They are recognized as cosmopolitan, since they have been found in association with marine, freshwater and terrestrial arthropods distributed worldwide (Contreras and Cafaro 2013). Initially, Trichomycetes were a class of fungi included within the phylum Zygomycota and divided into four orders: Eccrinales, Amoebidiales, Asellariales and Harpellales (Lichtwardt, 1986). Later on, with the help of molecular techniques, two groups (Amoebidiales and Eccrinales) were taken out

of the fungi and placed in the protists (Benny and O'Donnell 2000, Cafaro 2005, White et al. 2006). Together, they form an ecological group composed of Zygomycotan fungi (Asellariales and Harpellales) and Ichthyosporean protists (Amoebidiales and Eccrinales). These groups of microorganisms have evolved morphological adaptations such as cellular structures or secreted holdfast material to attach to the chitinous gut lining of their hosts (Valle and Cafaro 2008).

Within the order Eccrinales there are three families: Eccrinaceae, Palavasciaceae and Parataeniellaceae. The latter includes two genera: *Lajasiella* Tuzet & Manier ex Manier, 1968 and *Parataeniella* Poisson, 1929. There are only six species described for *Parataeniella*. Members of this genus have been found infecting the hindgut of terrestrial isopods. They reproduced asexually by means of uninucleate or binucleate secondary sporangiospores or primary uninucleate sporangiospores. They can either produce sporangia directly from their thalli that contain secondary binucleate infestation sporangiospores or the entire thallus can become a single sporangium that usually produces primary infestation uninucleate sporangiospores (Lichtwardt et al. 2001).

The Asellariales is probably the least studied order of trichomycetes, with a current record of 14 species. According to Valle (2006), the Asellariales include one single family, and three genera: *Asellaria*, *Orchesellaria* and *Baltomyces.* All three genera include filamentous species that reproduce asexually by arthrospore-like cells, which disarticulate from their corresponding thallus (Valle 2006). Within *Asellaria* Poisson 1932, there are 9 species described, and 3 of them have been found in Puerto Rico (*A. ligiae* Tuzet & Manier ex Manier, 1968; *A. dactylopus* Valle & Cafaro, 2008; and *A. jatibonicua* Valle & Cafaro, 2008). The genus *Asellaria* has filamentous, branched thalli that mostly consist of coenocytic cells prior to development of uninucleate arthrospores. The basal cell is morphologically distinct (Lichtwardt et al. 2001) and

is used as a structure for identification. The known species of *Asellaria* inhabit the gut of terrestrial, marine, and freshwater isopods. So far, very little has been studied about the relationship between *Asellaria* and its host, as cultivation methods have not been successful (Valle and Cafaro 2008).

The ecology of trichomycetes is poorly understood; again, since there are very few cultivable species. There are studies that have been performed concerning the prevalence (presence percentage) and seasonality of trichomycetes; however, all of them are related to aquatic hosts (Beard and Adler 2002; Beard et al. 2002; Beard and McCreadie 2003; Reeves 2004; Siri et al. 2008). In one of these mentioned studies the author suggested that the behavior of the trichomycetes depends on the biology of their hosts (Reeves 2004). Even though there are no similar studies made with terrestrial species we hypothesize that our trichomycetes under study will behave similar to those living in the gut of aquatic hosts. Additionally, that the prevalence of *Asellaria jatibonicua* will be higher in the most humid months of the year, since isopods tend to be highly sensitive to relative humidity.

Terrestrial isopods (Isopoda: Oniscidae) represents a good model host (since they are abundant organisms and possess a rapid growth rate) for the study of trichomycetes, since these organisms have evolved from marine environments and trichomycetes have been found present in isopods that inhabit different kinds of environments (marine, freshwater and terrestrial). As explain further in this manuscript, oniscidean isopods lacks special morphological adaptations that insects possesses **(**such as an external waxy layer in their cuticle**)** to resist changes in humidity, and the majority of these species represents important biological indicators of certain environmental variations (such as pollution and changes in temperature and humidity) (Paoletti and Hassall 1999; Hassall et al. 2010). These amazing organisms have evolved behavioral

adaptations (such as aggregation patterns and sheltering) that help them to survive the different environmental variations of the terrestrial environment (Hassall et al. 2010; Hornung 2011). The host species (*Litthorophiloscia culebrae*) we used in this study is poorly known, but our preliminary data have shown that it is very sensitive to variations in relative humidity. *L. culebrae* have been found present in various localities of Puerto Rico: Guajataca State Forest, Toro Negro State Forest, Rio Abajo State Forest, Parque de los Próceres in Mayagüez and the University of Puerto Rico, Mayagüez Campus. We intended to compare this host in the different places present in the island in order to evaluate if there are any trichomycete present inside their gut.

Since we found an urban locality (inside the University of Puerto Rico, Mayagüez Campus) in which *Littorophiloscia culebrae* was abundant, the main purpose of this study was to determine the seasonality and prevalence of *Asellaria jatibonicua* in this terrestrial isopod. Furthermore, the other localities in which the isopods were found were sampled tree times over the study period in order to evaluate the presence of trichomycetes and compare their abundance. Additionally, we intended to describe a new species of trichomycete in the genus *Parataeniella* that was found inhabiting the hindgut of the same host. We also provide a taxonomical key to the species of *Parataeniella* and a name suggested for the new species. As well, we include seasonality data for this new species.

2. LITERATURE REVIEW

2.1. Ecological aspects of the trichomycetes.

The trichomycetes are a group of microorganisms that live mostly as commensals in the gut of mandibulated arthropods. Generally found inhabiting herbivorous/detritivorous hosts and rarely predacious arthropods (Cafaro, 2000). This highly diverse group was, for a period of time, considered part of the lower fungi (Zygomycota). Current information indicates that these organisms comprehend an ecological group composed of Zygomycotan fungi (Asellariales and Harpellales) and Ichthyosporean protists (Amoebidiales and Eccrinales). These microorganisms where first discovered by Joseph Leidy in 1848 (Lichtwardt et al. 2001). Who subsequently described species of the now formally known genus *Enterobryus* of the Eccrinales. He first thought these microorganisms where a group of colorless algae. Time passed, confusion began and other scientists around the world continued discovering different species of this group and classifying them as algae, lower fungi or protists. It was not until 1948 (Lichtwardt et al. 2001) that this group was formally named Trichomycetes; referring to them as "hairy fungi" and then classified in the former phylum Zygomycota.

For some time, the group Amoebidiales was suspected not to be fungi (Lichtwardt 1986). Publications in the last 15 years indicate that the groups Amoebidiales and Eccrinales are part of the Ichthyosporean protists (Lichtwardt, 2008). These conclusions were reached after several DNA sequence analyses studies (Benny and O'Donnell 2000, Cafaro 2005, White et al. 2006). In addition, these analyses showed that these two groups are sister taxa (Figure 1) (Cafaro 2005). The other two groups of trichomycetes (Asellariales and Harpellales) were placed in the Subphylum Kickxellomycotina without designating a formal class (Figure 2) (Hibbett et al.

2007); hence, the term trichomycetes (lower case t) has no classification meaning and it is considered an ecological assembly rather than a phylogenetic group.

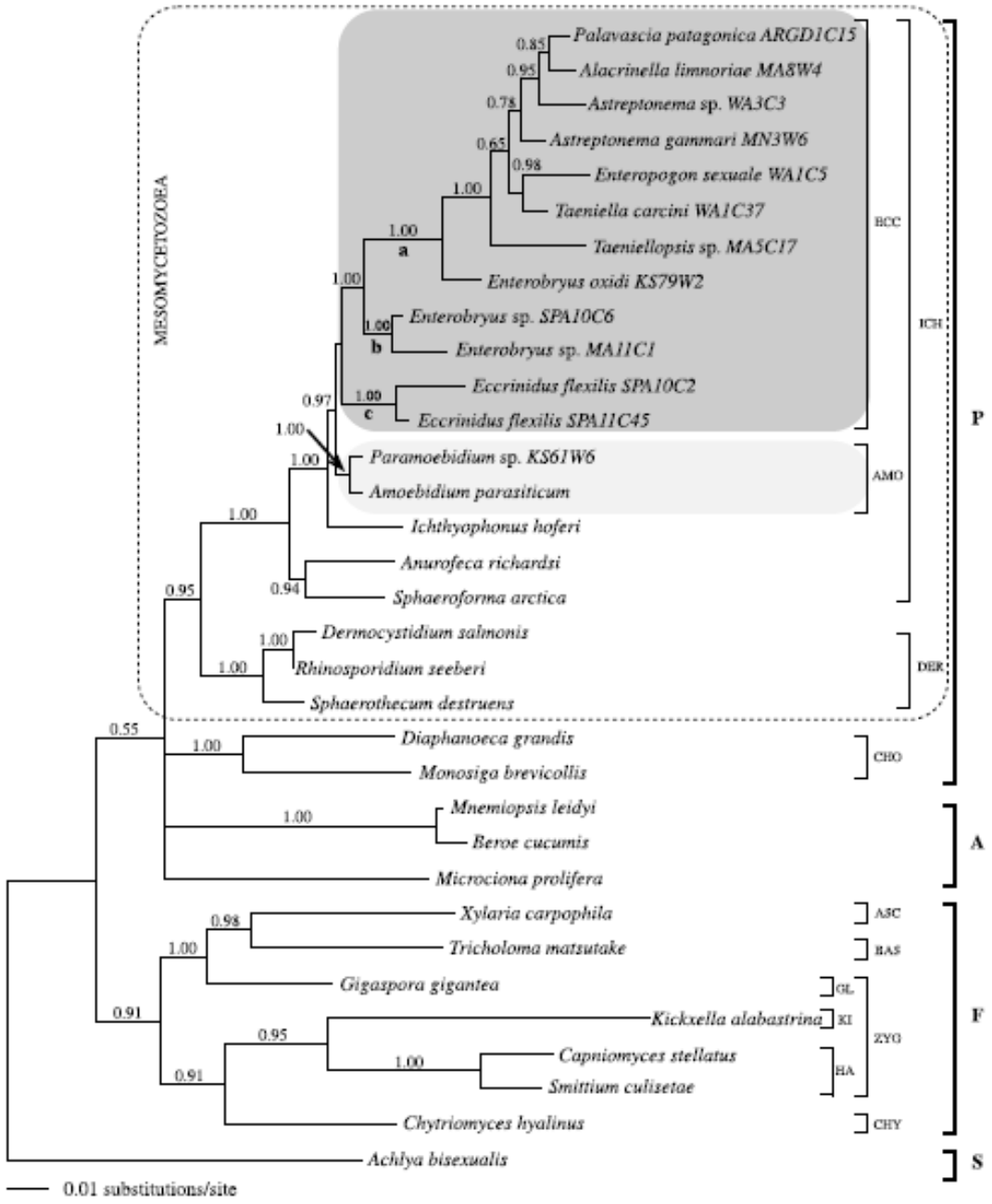

Figure 1. Bayesian phylogenetic tree from 18S dataset showing the position of Eccrinales and Amoebidiales according to Cafaro (2005). ECC, Eccrinales; AMO, Amoebidiales; DER, Dermocystidia; ICH, Ichthyophonida; CHO, Choanoflagellates; GL, Glomales; KI, Kickxellales; HA, Harpellales; ASC, Ascomycota; BAS, Basidiomycota; ZYG, Zygomycota; CHY, Chytridiomycota; A, Animalia; F, Fungi; S, Stramenopila; and P, Protista.

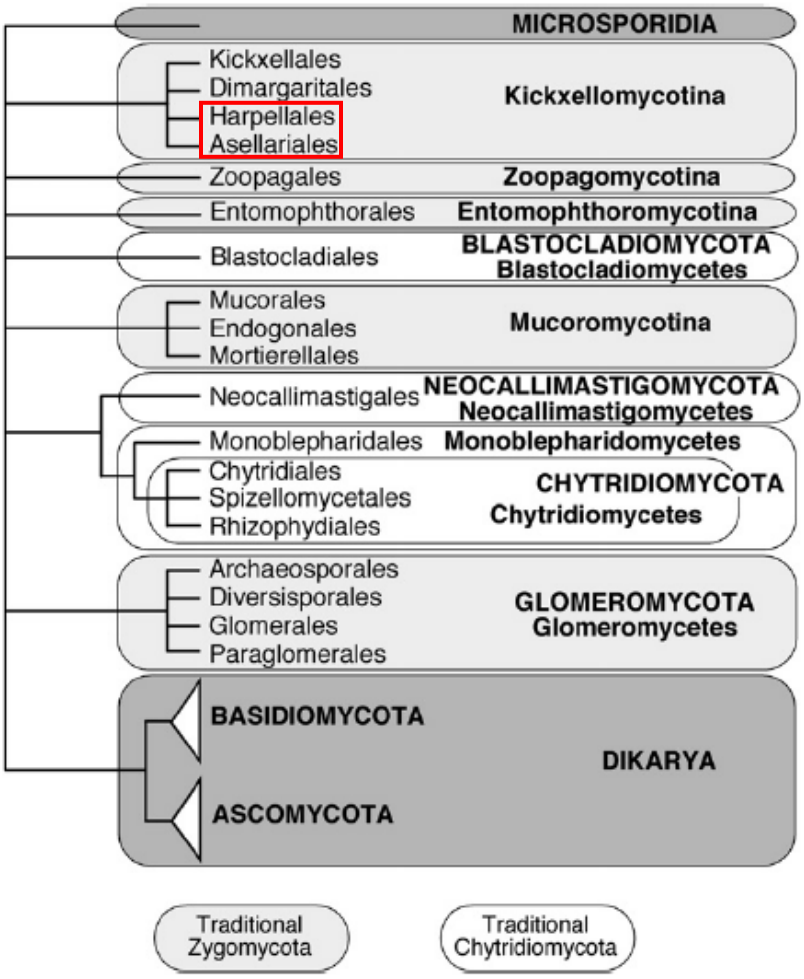

Figure 2. Phylogenetic tree for the classification of the Basal Fungi and Dikarya according to Hibbett et al. (2007); indicating the position of the Harpellales and Asellariales until 2007.

2.2. Historical perspective about the classification of trichomycetes.

According to Lichtwardt et al. (2001), the trichomycetes studies can be divided into four historical periods, which are mainly defined by the contributions of individual investigators with new discoveries and emphases within each time period. Here is a summary of each period with the most relevant information available and already published by Lichtwardt et al. (2001).

The first period (1848 - 1904) started with the discovery of several species of Eccrinales by Joseph Leidy. The descriptions of these species were later published under the generic name

Enterobryus. Trichomycetes were first thought to be colorless algae. Later, in 1853, Charles Robin discovered in France another species of *Enterobryus,* and thought they were related to the fungal order Saprolegniales. After that, a different kind of arthropod associate was discovered in Europe by Lieberkühn and Schenk, which lived in the exoskeleton of different aquatic arthropods, which was later named *Amoebidium parasiticum* by Cienkowski, 1861 (Grigg and Williams 1990). Different scientists of that period thought this organism was an algae, a lower fungi or even a protozoan. Culminating this first period, in 1895 Hauptfleish described a second genus of Eccrinales, namely, *Astreptonema.* This organism was discovered inhabiting the hindgut of an amphipod and was also thought to belong to the Saprolegniales.

The second period (1905 - 1928) continued with the discovery of numerous other Eccrinales and was started with morphological and taxonomic studies of eccrinids from marine crustaceans, millipedes and hydrophilid beetles. Later, the most extensive investigations of this period were experimental studies on the biology of *Amoebidium* species, including host specificity and the effects of environmental factors on the development of their morphological characters. Throughout 1927 and the mid-1930's, Raymond Poisson commenced describing new genera and species of eccrinids from amphipods and isopods and expanded the study of trichomycetes by including other groups of arthropods. By the end of this period there was already a shared belief that the trichomycetes were some kind of fungi.

The beginning of the third period (1929 - 1959) was marked by the discovery of the first Harpellales (*Harpella melusinae*) by Léger and Duboscq. In this same period the only endocommensal genus of Amoebidiales, *Paramoebidium,* was named, while Poisson discovered and described the eccrinid genus *Parataeniella* and another genus placed in the order Asellariales, named *Asellaria.* This period was in its majority significant for the extensive

description and extension of species known and their host types. In addition, many scientists studied their morphology and ecology, as well as their worldwide distribution. Here the term "Trichomycetes", was introduced to refer to this group that was in the past classified as fungi.

The last of the four periods described by Lichtwardt et al. (2001), the fourth period (1960 - 1985), was one of much change and better comprehension in the studies of trichomycetes. The first successful isolation in axenic culture of a trichomycete (*Amoebidium parasiticum*) from the exoskeleton of an arthropod, which allowed experimental studies toward a better understanding of these species, was accomplished by Whisler (Whisler 1960, 1962). Then, other scientists isolated different strains of many species of the order Harpellales that we now know as members of *Smittium*. The availability of these isolations permitted different kinds of experimental studies that helped comprehend the symbiotic relationship with their hosts and the biology of these organisms. In consequence, it was discovered that *Smittium morbosum* was lethal to mosquito larvae and that the method of dispersion of *Harpella melusinae* was also pathogenic to different adult blackflies. These discoveries demonstrated that trichomycetes were not necessarily commensalistic all the time. In addition, other scientists demonstrated that that trichomycetes can be beneficial to their hosts when deprived of certain nutrients. This was assured by experiments with *Smittium culisetae* infecting the mosquito *Aedes aegypti* (Horn and Lichtwardt, 1981).

After 1985, the studies on trichomycetes have been upgraded by the different publications available and the extensive comprehension studies that have been made to date. Before this "fifth period" we knew the Trichomycetes (with uppercase T) as a whole fungal group. Currently, we classify two of its orders (Amoebidiales and Harpellales) as protists and the other two (Asellariales and Harpellales) as fungi. These conclusions were made after molecular and phylogenetic studies. Now, when we refer to this group it is mentioned as trichomycetes

(with lowercase t), referring to them as an ecological group and not a phylogenetic one (Lichtwardt 2008, 2012).

2.3. The symbiotic relationship of trichomycetes.

The nature of symbiotic associations between two evolutionary distinct species is influenced by biotic (e.g., food availability, host species physiological condition, host preference) and abiotic factors (e.g., temperature, pH, humidity) of the environment where the interaction occurs (Kim, 2005). Experimental studies on the symbiotic relationship between trichomycetes and their hosts have been limited, since very few species have been successfully cultured (Lichtwardt, 2001).

Certain Harpellales inhabit the gut of hosts that can be vectors of different kinds of pests and diseases such as mosquitoes (Culicidae), blackflies (Simuliidae), and biting midges (Ceratopogonidae) (Lichtwardt 2012). Most of the time trichomycetes have been considered as commensal symbionts. However, these amazing organisms may provide specific benefits to its host, such as the survival to vitamin and sterol deficient environments (Horn and Lichtwardt 1981), considering this relationship to be mutualistic. Furthermore, a fungal member of the trichomycetes, *Smittium morbosum*, was found to be lethal to mosquito larvae (Sweeney 1981). The Harpellales include other fungi that as a method of dispersion invade the developing ovaries of blackfly larvae resulting in the sterility of the adult female whose eggs have been replaced by fungal cysts (Labeyrie et al. 1996).

There are two species of Harpellales (*S. culisetae* and *S. culicis*) that are known to benefit their hosts under specific environmental conditions or deprivation of certain nutrients (Horn and Lichtwardt 1981). These two organisms are shed with the gut cuticle at the time of molting and

are thought not to be detrimental to the host at any stage of its life cycle (Lichtwardt and Arenas 1996). In contrast, *S. morbosum* remains within the host at the time of molting, and sometimes persists through the adult stages, causing blockage of the gut and the consequent death of its host (Sweeney 1981).

There is a great deal of information concerning the taxonomy of trichomycetes, however, information regarding their ecology is scarce. Furthermore, the most studied organisms included in this group (in their recent ecological perspective), inhabit freshwater or marine hosts (Grigg and Williams 1989, Taylor et al. 1996, Labeyrie et al. 1996, Beard and Adler 2002, Beard and McCreadie 2002, López Lastra et al. 2003, Beard et al. 2003, Reeves 2004, Siri et al. 2008). Yet, terrestrial hosts have not been extensible studied regarding the ecology of its symbionts.

2.4. Ecology of terrestrial isopods.

The order Isopoda contains over 10,300 marine and freshwater species (Wilson 2008) and more than 3,600 terrestrial species (Schmalfuss 2003). These organisms have evolved in parallel with insects from the same aquatic ancestor (Regier et al. 2010). Though, the physiological adaptations of terrestrial isopods to land life seem incomplete, since they do not possess a waxy cuticle to prevent desiccation (Hadley and Quinlan 1984).

Terrestrial isopods constitute the suborder Oniscidea, which is the single suborder of Crustacea almost exclusively composed of strictly terrestrial species (Broly et al. 2013). They share the same ecological niches (uppermost soil layers and leaf litter) and similar lifestyles with many insects and millipedes (David and Handa 2010). The ecological distribution of oniscideans ranges from supralittoral zones far into dry land, from sea level to high mountains and caves (Hornung 2011). Furthermore, terrestrial isopods are primarily detritivorous organisms that feed

on leaf litter, decayed wood, fungi and bacteria (Broly et al. 2012), and constitute one of the most important groups of organisms driving the dynamics of soil (Hassal et al. 1987; Zimmer 2002; Broly et al. 2012). According to Paoletti and Hassall (1999), isopods are more abundant in calcareous soils rather than acid ones for their requirement for calcium in their exoskeletons. They often use coprophagy as a method of nutrient uptake improvement (Paoletti and Hassall 1999). During their evolutionary history, they have developed different morphological, ecological and behavioral adaptations to the terrestrial ways of reproduction, respiration, excretion and protection against desiccation (Hornung 2011). Terrestrial isopods are the only group of Crustacea that can live on land throughout their complete life cycle (making them a good model host for the study of trichomycetes); however, they are able to survive land conditions by evolving patterns of behavior, restricting them to moist dark habitats and by avoiding the typical desiccating land conditions (Waloff 1941).

These organisms are beneficial for their role in enhancing nutrient cycling, by comminuting of debris and transporting it to the moister microsites in soil (Paoletti and Hassall 1999), and also for transporting propagules of bacteria, fungi and vesicular arbuscular mycorrhizae through soils (Rabatin and Stinner 1988). Moreover, terrestrial isopods usually respond easily to environmental contamination and impact, with increased mortality, loss of biomass and a decrease of number of species, resulting from serious levels of pollution (Paoletti and Hassall 1999). Isopods are capable of accumulating high levels of heavy metals (such as: copper, zinc, lead and cadmium) in vesicles such as lysosomes (Wieser et al. 1977; Paoletti et al. 1988; Prosi and Dallinger 1988). For this reason, they have been very useful for monitoring heavy metal pollution in industrialized and urbanized areas (Paoletti et al. 1988; Dallinger et al. 1992). According to Paoletti and Hassall (1999), since isopods are also predated by a wide range

of other vertebrates and invertebrates, their bioaccumulation of toxic materials can also have ramifications for trophic levels higher up the food chain. Since these organisms are extremely sensitive to environmental changes they need to be study in every aspect of its ecology for a further understanding of their behavior. Our study species (*L. culebrae*) for example is a poorly studied, but very abundant in Puerto Rico making it a good model for the studies of trichomycetes in different sites of the island.

2.5. The digestive system of terrestrial isopods.

According to Hames and Hopkin (1989), the digestive system of terrestrial isopods is divided into five regions: the foregut (1), the anterior chamber (2), papillate region (3) the rectum (4) of the hindgut, and the hepatopancreas (5), which opens into the foregut and consists of four blind-ending tubules. They also mention the presence of a powerful muscular sphincter between the papillate region and the rectum.

Food is masticated by the mandibles of the isopod, which is then pushed into the oesophagus to the proventriculus of the foregut where they are briefly fragmented before passing rapidly into the anterior chamber of the hindgut (Hames and Hopkin 1989), remaining there for up to seven days (Hassall and Jennings 1975). When the hindgut is full of food, liquids and food particles are forced back into the foregut via the typhiosole channels; this material is then filtered in the foregut and passed into the lumen of the hepatopancreas where further digestion and absorption of nutrients takes place (Hames and Hopkin 1989). Finally, remaining material in the hindgut passes into the rectum where faecal pellets are compacted before further expulsion (Hames and Hopkin 1989).

According to Lichtwardt et al. (2001), the complete assimilative stage of trichomycetes takes place within the gut, where they obtain all sustenance from material present in or passing through the gut lumen. Most trichomycetes (including the two species studied in this research) inhabit the hindgut, where absorption of food is said to be minimal; in consequence, it is assumed that these organisms do not deprive their hosts from nourishment, although there is no evidence supporting this conclusion (Lichtwardt et al. 2001). However, the same authors state that it is often the "healthy" populations of arthropods that seem to be highly infested, but there are no comparative studies performed that support this perception. Our data have shown the same results: the isopods with or without the presence of trichomycetes seem to be the same in body measurements, sensitivity to relative humidity variations and type of movement. This suggests that at least species within the genus *Asellaria* may not have any effect on its host and represents a commensalistic association as Lichtwardt et al. (2001) also suggests.

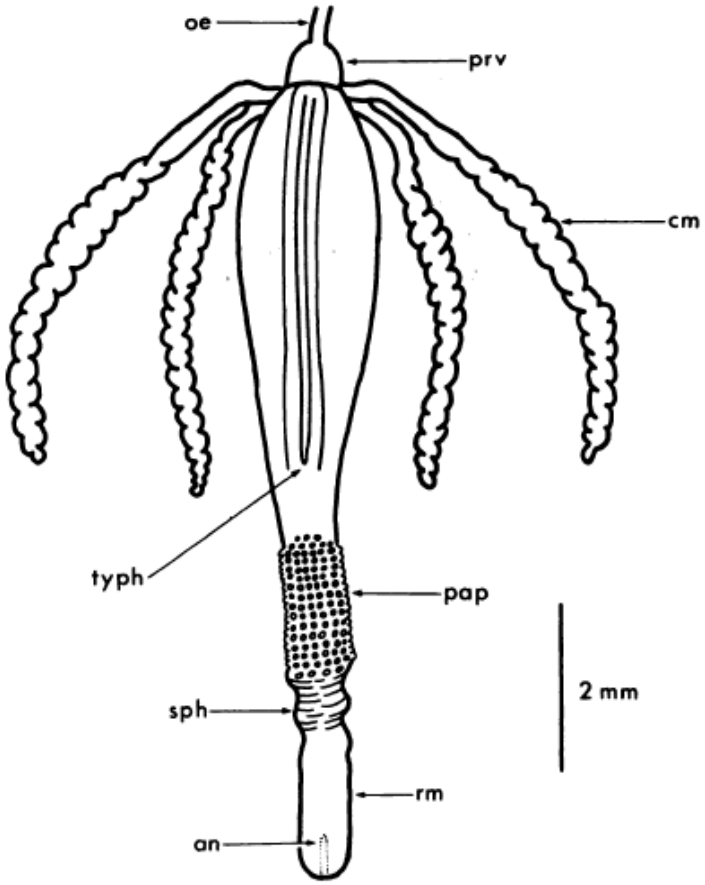

Figure 3. Schematic diagram of the digestive system of *Philoscia muscorum* according to Hassall and Jennings (1975). cm, caecum (midgut); oe, oesophagus; pap, papillate region of hindgut; prv, proventriculus; rm, rectum; sph, sphincter; typh, typhiosole; an, anus.

2.6. Description of *Litthorophiloscia culebrae*

The terrestrial isopod *Litthorophiloscia culebrae* is included in the phylum Arthropoda, class Crustacea, order Isopoda, suborder Oniscidea, and family Halophilosciidae. It was first described as *Philoscia culebrae* by H. Moore (1901) from Culebra Island (Schmidt and Leistikow 2004). In Culebra Island the vegetation is mostly deciduous (The deciduous plants are those that are leafless for a certain period of time each year, usually during the cold season) and species are spiny, with leaves small and succulent (succulents are plants that store water in their

leaves, stems or roots); consisting of dense shrubs, small shrubs, pastures and vegetation of semi-humid forests (Dubón, 2013). *L. culebrae* a wide distribution, having been recorded from Florida, Puerto Rico, Hawaii, Cuba, Virgin Islands, Canary Islands, Angola, Madagascar, and Yemen Brazil (Schmalfuss 2003; Taiti and Ferrara 2004, Araujo and Taiti 2007). Moreover, the genus *Litthorophiloscia*, with 21 species, has mainly a tropical distribution (Taiti and López 2008). This species is fully described in Araujo and Taiti (2007) from collected samples from Brazil. *Litthorophiloscia culebrae* presents a yellowish color with brown spots (Figure 4, 5). The maximum length in males is 2.5 mm and females with marsupial pouch can reach 3.6 mm.

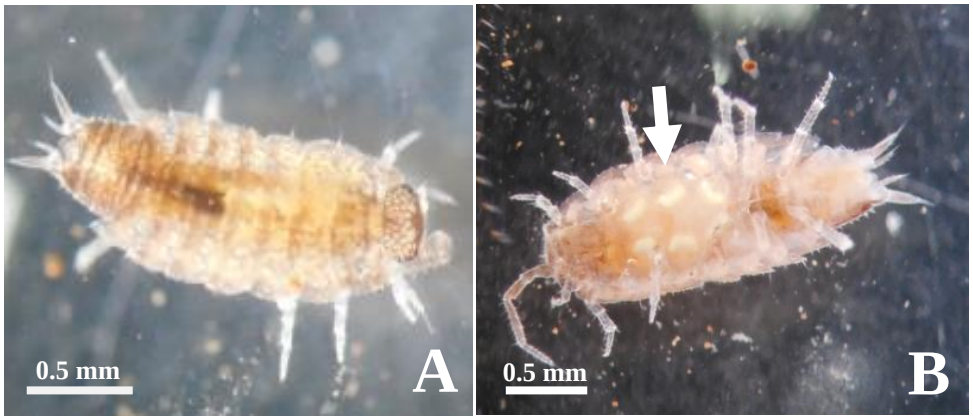

Figure 4. Images of *Litthorophiloscia culebrae* taken under an Olympus stereomicroscope. A. Posterior view of isopod whole body, B. Anterior view of isopod whole body with perception of the marsupial pouch with eggs (arrowed).

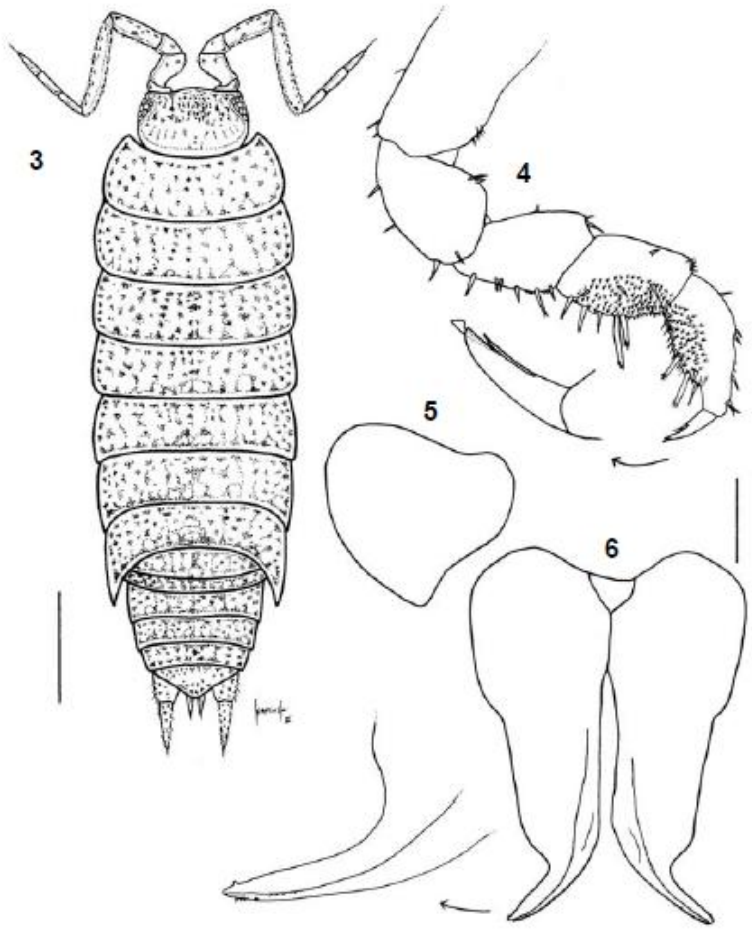

Figure 5. Description of *Litthorophiloscia culebrae* according to Araujo and Taiti (2007): A- ♂; B- pereiopod 1; C- pleopod 1 exopod; D- pleopod 1 endopod. Scale bars: (A) = 1mm; (B-D) = 0.1mm.

3. MATERIALS AND METHODS

3.1. Study area description.

The principal sampling area of the present study is located in an urban area inside the University of Puerto Rico, Mayagüez Campus. It is specifically located in front of Jesús T. Piñero's Building (18°12'38.3" N - 67°08'35.4" W) (Figure 6) under a *Calophyllum brasiliense* tree. This area was selected since the publication of the description of *Asellaria jatibonicua* (Valle and Cafaro, 2008) mentioned that there was a population of *Litthorophiloscia culebrae* inside the University of Puerto Rico, Mayagüez Campus. This publication also mentioned populations of this host in other localities of Puerto Rico, such as Parque de los Próceres in Mayagüez and State Forest of Toro Negro in Jayuya. These other two localities were also sampled; however, *A. jatibonicua* was never found in any of the sampled isopods. Additional localities were also sampled: Guajataca State Forest in which *A. jatibonicua* was present in every occasion sampled and Rio Abajo State Forest in which *A. jatibonicua* was never present.

Figure 6. Sampling area map in which 10 isopods of *Litthorophiloscia culebrae* were collected each week for 18 months in order to evaluate the presence of *Asellaria jatibonicua*. A- Caribbean; B- Puerto Rico; C- Specific sampling area inside the University of Puerto Rico, Mayagüez Campus. Images extracted from Google™ Earth.

3.2. Host collection and dissection.

Isopods were collected by hand from under decayed vegetation and preserved in plastic containers with moist vegetation and soil to avoid desiccation. The living isopods were dissected the same day of collection (to maintain an intact gut at the moment of dissection) under a stereomicroscope with the help of thin forceps and needles for extracting the gut, cleaning it in a drop of water and isolating the fungal thalli, which were then transferred into a clean slide with distilled water. The slide was then covered and observed on a phase contrast microscope for identification. Specimens were fixed and stained by infiltration with Lactophenol Cotton Blue (LPCB) and sealed with clear fingernail polish. Water-mounted and stained slides were photographed with a digital camera in an Olympus CX41 compound microscope. Additionally, the number of infested isopods was recorded for each collection day. In order to observe if there was a seasonal pattern or fluctuation in the number of isopods, each week from August 2012 to January 2014, 10 isopods were collected in the morning at random in a grid of 10 m X 10 m previously divided in sections of 1 m X 1 m.

Figure 7. Protocol for the collection and processing of isopods in order to evaluate the presence of trichomycetes. A- Host collection within litter by hand with the help of small shovels; B- Needed laboratory equipment for the isopods dissections and trichomycetes' glass slides preparations (stereomicroscope, distilled water, thin needles, forceps, glass slides, coverslips, Lactophenol Cotton Blue, clear nail polish); C- Hosts dissection, extraction of gut from the isopod's body using a stereomicroscope and thin needles and forceps; D- Preparation of slides with trichomycetes (arrowed) by infiltration with Lactophenol Cotton Blue.

3.3. Exposure of isopods to different percentages of relative humidity.

In order to predict population responses to humidity variations, isopods were collected and placed in 200 ml containers tightly closed with plastic lids (10 isopods per container). An open-top plastic sample vial containing 25 ml NaCl solution was placed in the center of each container (Figure 8) to provide a specific relative humidity (RH) (Table 1). The isopods were left inside each chamber without any food. Activity of the isopods was assessed by classifying them

as either 0 (inactive), 1 (active) after tapping the container gently to stimulate movement. This procedure was performed three times. These methods were performed according to procedures described by Sjursen et al. (2001).

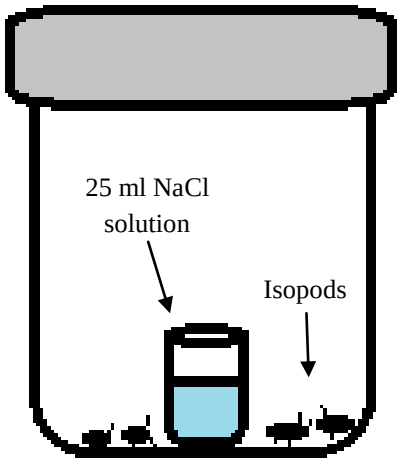

Figure 8. Schematic view of isopods inside plastic closed chamber with glass vial inside containing NaCl solution with a specific concentration in order to create a specific relative humidity.

Table 1. NaCl concentrations needed to create the corresponding relative humidity (RH) in each 200 ml closed chamber. As described in Sjursen et al. (2001).

g NaCl/L dH2O	RH (%)
0	100
31.60	98.2
53.80	97.0
62.50	96.5
71.20	96.0
81.20	95.5
90.34	95.0
99.40	94.5
108.46	94.0
117.52	93.5
126.57	93.0

3.4. Fluctuations in number of isopods in population through time.

The 10 m X 10 m sampling grid was divided in square blocks of 1 m X 1 m. In each sampling day there was a random selection of tree squares. Each square was divided into squares sections of 0.25 m X 0.25 m. In each 1 m X 1 m square selected there was an additional random selection of tree 0.25 m X 0.25 m squares (Figure 9) from which we collected litter and soil in 500 cm^3 glass containers. The material was then transported to the laboratory in plastic containers. The isopod extraction from litter and soil was performed by hand. After counting the isopods collected they were returned to its original sampling place. The samples were taken at monthly intervals from August 2014 through February 2015. Samples were collected in the early morning where the temperature was presumed to be lower than during other times of the day.

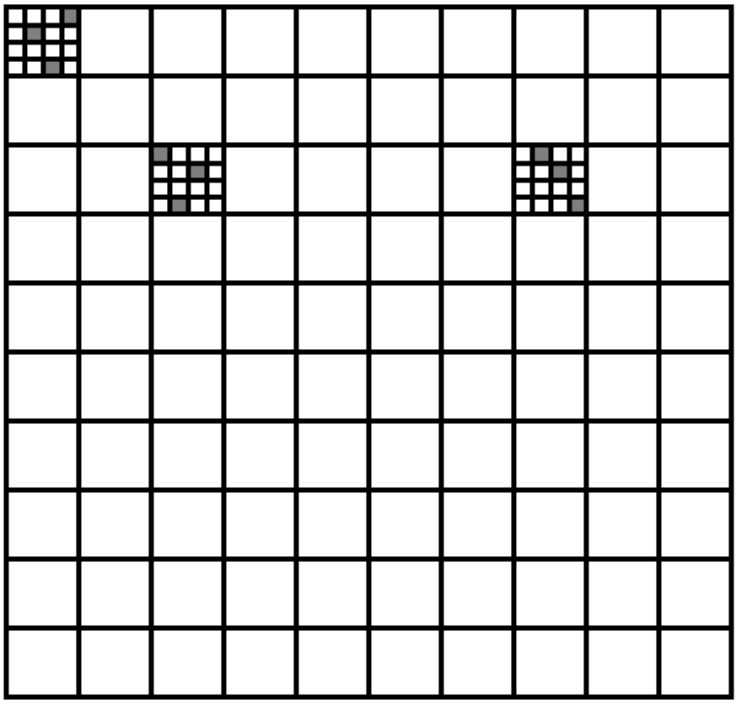

Figure 9. Schematic view of sampling area in order to evaluate the fluctuation in the number of individuals of the isopod *L. culebrae* through time. The 10 m X 10 m grid was divided in sections of 1 m X 1 m. Each 1 X 1 section was subsequently divided in smaller sections of 0.25 m X 0.25 m.

3.5. Isopod growth until adult stage.

Female isopods were identified as being in their reproductive period after perception of eggs inside the marsupial pouch. After this determination, their body length was measured. Subsequently, each female was placed in a humid Petri dish with litter extracted from their natural habitat and every egg inside its pouch was counted. Afterward, the microcosm was monitored daily to enumerate emerging isopods and to measure their body length. This assessment was done every two weeks until adult stage.

3.6. Statistical methods.

To establish a possible relationship between environmental factors (relative humidity, temperature and precipitation) and trichomycete prevalence a linear regression was performed. In addition, a linear regression was performed to establish if there was a relationship between the length of the female progenitors and the isopods they procreated. Furthermore, to establish if there was a significant change in the isopod numbers in the population over time and to assess prevalence variation analyses of variance (ANOVA) and Tukey tests were carried out. All the statistical data was evaluated using an Error Type I (α) of 0.05. The assumption of "normality" was examined for all the data with a Shapiro-Wilks (modified) Normality test. All the statistical tests were performed using the statistical program InfoStat (2008 version). In addition, a One-way repeated measures ANOVA was performed using the statistical program SPSS to assess if there was a significant difference of prevalence between and within months for both trichomycetes.

4. RESULTS

4.1. Identification of *Asellaria jatibonicua*

The species included under the genus *Asellaria* are mostly identified by their morphological structures. This genus has filamentous, branched thalli that typically consist of coenocytic cells prior to development of uninucleate arthrospores. The basal cell is morphologically distinct and is used as the principal structure of identification. The known species of *Asellaria* inhabit the gut of terrestrial, marine, and freshwater isopods. In the present study it was presumed that *Asellaria jatibonicua* was the only species of *Asellaria* inhabiting the hindgut of *Litthorophiloscia culebrae* (host under study), since the publication of this species description (Valle and Cafaro 2008) stated that it was found in this specific host in different areas of Puerto Rico. Additionally, it appears that species of *Asellaria* maintain a species-specific behavior (personal observation), since near populations of other species of isopods have been dissected and have not manifested the presence of *A. jatibonicua*.

The thalli of *A. jatibonicua* were found in the terminal portion of the hindgut of *L. culebrae* between August 2012 and January 2014. The specimens presented highly branched and septate thalli, being the longest those branches that protrude from the basal cell. Basal cell has an elongated form, 50-115 l X 15-20 w µm, with a swollen or campanulate terminal portion that secretes a sticky holdfast substance that facilitates the attachment to the lining of the hindgut. Its entire thallus measured 500-1000 l X 10-15 w µm and its mature arthrospores 15-35 l X 6-12 w µm. The morphological measurements of this trichomycete coincided with the descriptions of Valle and Cafaro (2008). However, these authors mentioned the presence of zygospores and conjugating tubes, which were not observed in any of our samples along the study period.

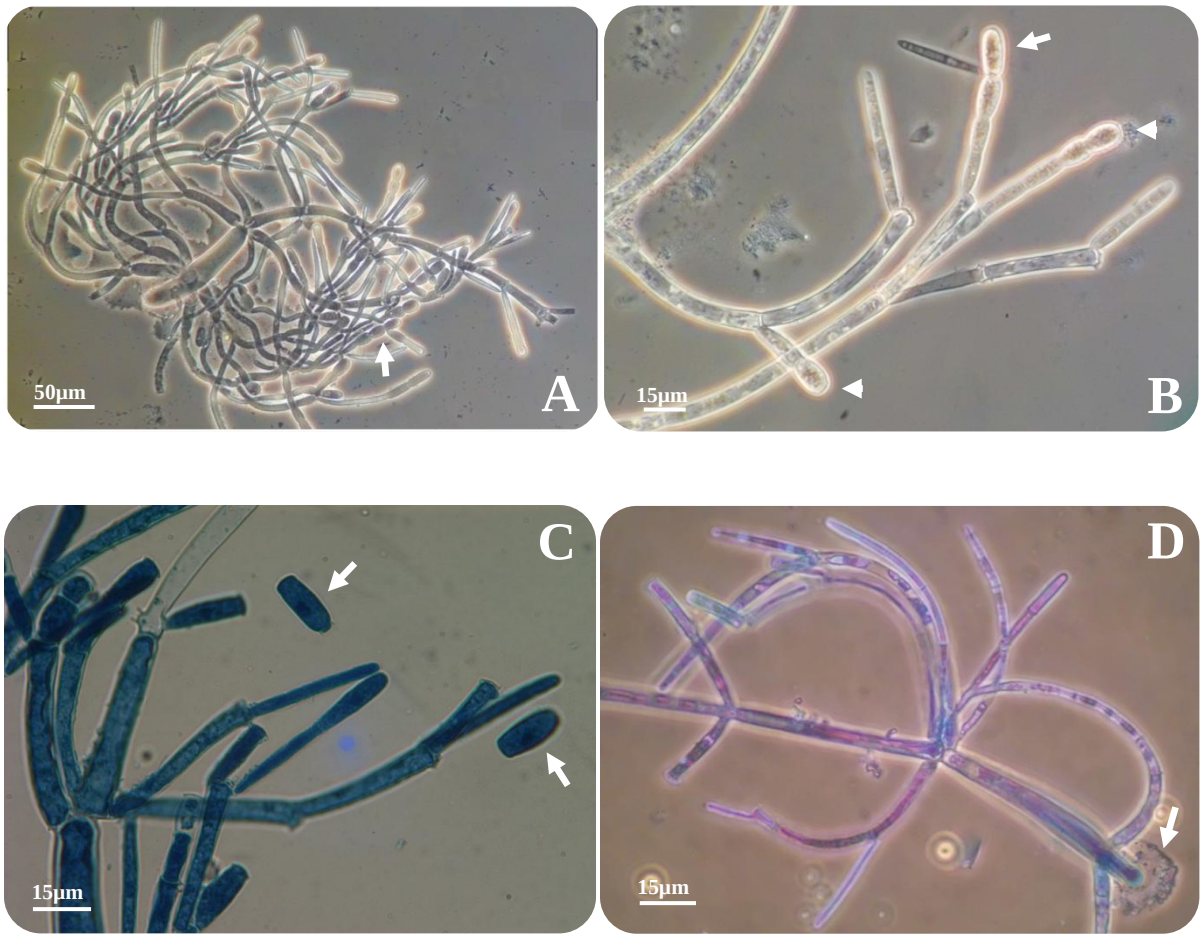

Figure 10. Morphological characters of *Asellaria jatibonicua* found in the terrestrial isopod *Litthorophiloscia culebrae.* Images were taken with an Olympus CX41 phase contrast microscope A. Thallus overview with fertile branches and intercalary arthrospores (arrow). B. Terminal branches with immature arthrospores (arrowheads) and mature arthrospores detached from terminal branches (arrow). C. (LPCB) Detached arthrospores from terminal branches (arrows). D. (LPCB) Thallus in which the basal cell with holdfast and holdfast secretion (arrow) is observed as well as the basal branches.

4.2. Identification of a new *Parataeniella* Poisson species.

During this study a new species of trichomycete was discovered inhabiting the hindgut of *L. culebrae*. The name *Parataeniella limonispora* was suggested for this new species and a description is provided together with a key to all the species of *Parataeniella* Poisson.

Parataeniella limonispora Rivera-Beede and Cafaro, sp. nov. Figure 8.

Thallus up to 150 µm long by 6-9 µm wide, cylindrical, non branched, multinucleated, hyaline, cytoplasm granulose; entire thallus becoming a single sporangium. Thin-walled primary sporangiospores, limoniform, uninucleate, 12-15 µm long by 6-9 µm wide, usually 2 to 14 per sporangium. Secondary sporangiospores not observed. Spore mother cell not persistent. Holdfast campanulate, 2-4 µm long by 5-9 µm wide, attached to hindgut lining of Isopoda.

Etymology. Lemon shaped, referring to the morphology of the sporangiospores. Epithet derived from *Citrus limon* (lemon fruit).

Specimens examined. Puerto Rico. Mayagüez: Urban area in front of Jesus T. Piñero's building (under a *Calophyllum brasiliense* tree) at the University of Puerto Rico, under vegetation debris, prepared from *Litthorophiloscia culebrae* (Moore, 1901) (Isopoda: Oniscidae).

Key to species of *Parataeniella* (Poisson, 1929) (Ichthyosporea: Eccrinales)

Thalli unbranched, can either produce a series of sporangia with single binucleate secondary infestation sporangiospore, or the entire thallus becomes one sporangium with many uninucleate or binucleate primary infestation sporangiospores (Lichtwardt et al. 2001). Attached to the hindgut lining of terrestrial Isopoda (Crustacea).

1 Thalli short, up to 150 µm long x 3.5-9 µm wide 5

1’ Thalli up to 300-385 µm long, 12-15 µm wide 6

2 Thalli dimensions 210-690 x 6-21 µm, binucleate thin walled secondary sporangiospores, binucleate thick walled primary sporangiospores oval to ellipsoidal, uniseriate within sporangium, 15-24 x 7-12 µm . . . . . ***P. flavospora***

3 Thalli dimensions 170-180 x 10-12 µm . . . . . ***P. mercieri***

4 Thalli short to 2 mm, binucleate secondary sporangiospores 40-70 x 15-19 µm; primary sporangiospores, uninucleate, oval to angular, 31-35 x 15-18 µm . . . . . ***P. latrobi***

5 (1) Primary infestation sporangiospores binucleate, uni- or biseriate . . . . . ***P. scotonisci***

Primary infestation sporangiospores uninucleate and uniseriate ... ***P. limonispora* sp. nov.**

6 (2) Secondary sporangiospores 45-50 µm long; uni- or binucleate primary sporangiospores, oval to ellipsoidal 15-35 µm long . . . . . ***P. dilatata***

Secondary sporangiospores, binucleate, ~12 x 10 µm; primary sporangiospores, ~11 in diameter, globose to angular in shape, biseriate, rarely uniseriate . . . . ***P. armadillidii***

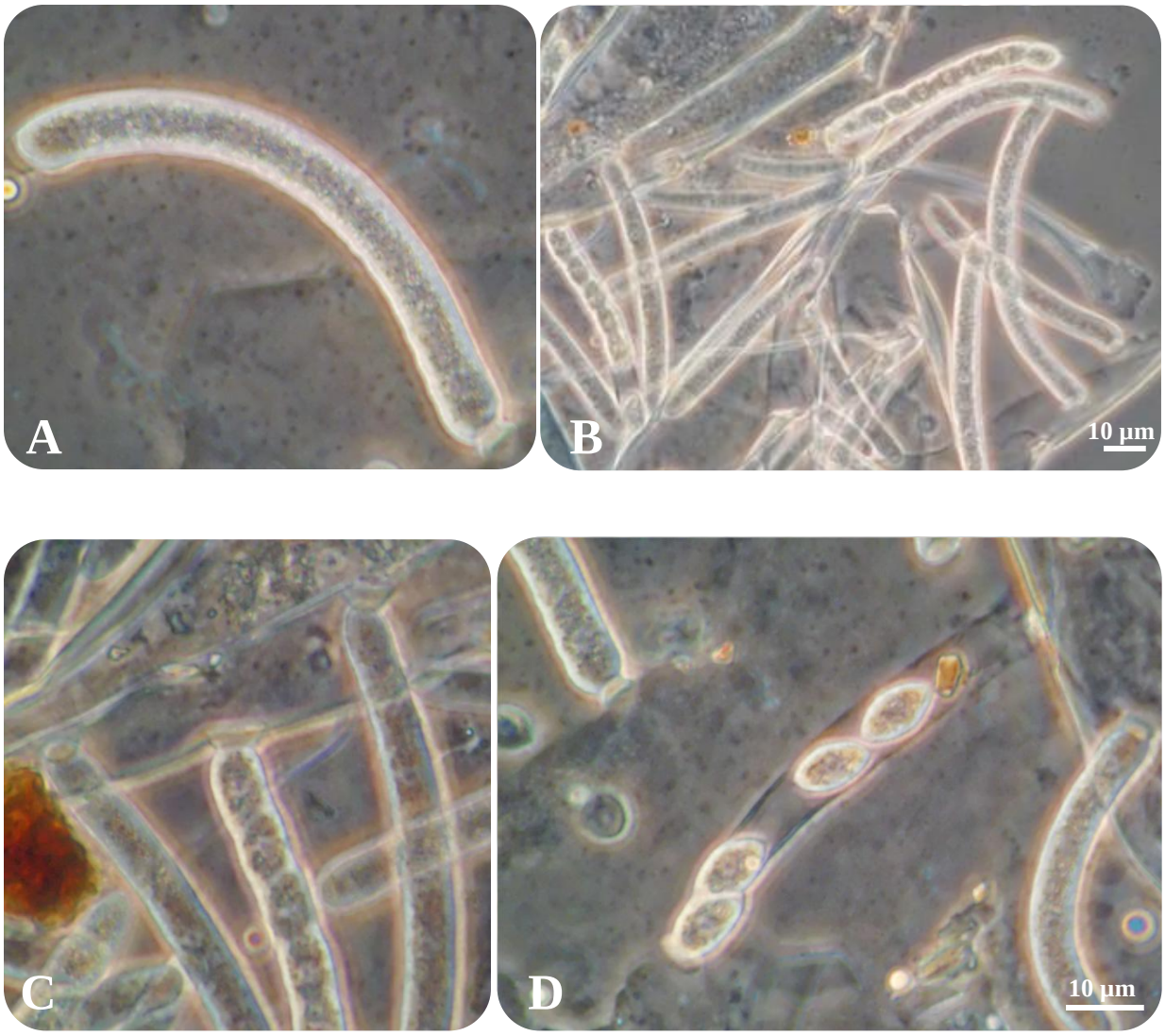

Figure 11. Morphological characters of *Parataeniella limonispora* sp. nov. isolated from *Litthorophiloscia culebrae*. **A** - Zoom to a single immature thallus attached to the gut lining of *L. culebrae* by a campanulate holdfast; **B** - Thalli attached to the gut's lining by campanulate holdfasts; **C** - Thalli showing campanulate holdfasts and thallus showing a differentiating stage of uninucleate sporangiospores inside sporangium; **D** - Thallus converted into one sporangium with mature uninucleate sporangiospores being released through an apical aperture of the thallus.

4.3. Prevalence and seasonality of *A. jatibonicua* and *P. limonispora* sp. nov.

On average, 26% of the isopods (N=750) were infested with *A. jatibonicua* and had a minimum prevalence of 7% in August 2012 and a maximum of 54% in December 2012 (Table 2; Figure 12, 13); however, this peak was not repeated over the study. The prevalence did not increase through time, although it fluctuated suggesting a relationship to different regimes of relative humidity (Figure 15). As the relative humidity increased the prevalence did as well and in the opposite situation the prevalence of the trichomycete diminished. It is important to point out that *Parataeniella limonispora* sp. nov. showed similar seasonal behavior and relationship with relative humidity (Figure 16). On average, 19% of the isopods (N=750) were infested with *Parataeniella limonispora*. The prevalence showed a minimum of 0% in August 2012 and maximum of 60% in December 2012 (Table 3; Figure 12, 14). The relationship of these two species seems to be tightly associated to the biology of the host.

Table 2. Descriptive statistics for the prevalence of *A. jatibonicua* and fluctuations of: relative humidity, average precipitation and temperature; N = 18 (correspond to the number of months sampled).

Variable	Mean	S.D.	CV	Minimum	Maximum
Prevalence %	0.26	0.12	44.70	0.07	0.54
RH %	74.61	8.92	11.95	64.00	99.00
Avg Precip. (mm)	0.36	0.18	50.92	0.11	0.61
Temperature (°C)	31.25	1.54	4.93	29.00	34.30

Table 3. Descriptive statistics for the prevalence of *P. limonispora* sp nov. and fluctuations of: relative humidity, average precipitation and temperature; N=18 (correspond to the number of months sampled).

Variable	Mean	S.D.	CV	Minimum	Maximum
Prevalence %	0.19	0.17	89.57	0.00	0.60
RH %	74.61	8.92	11.95	64.00	99.00
Avg Precip. (mm)	0.36	0.18	50.92	0.11	0.61
Temperature (°C)	31.25	1.54	4.93	29.00	34.30

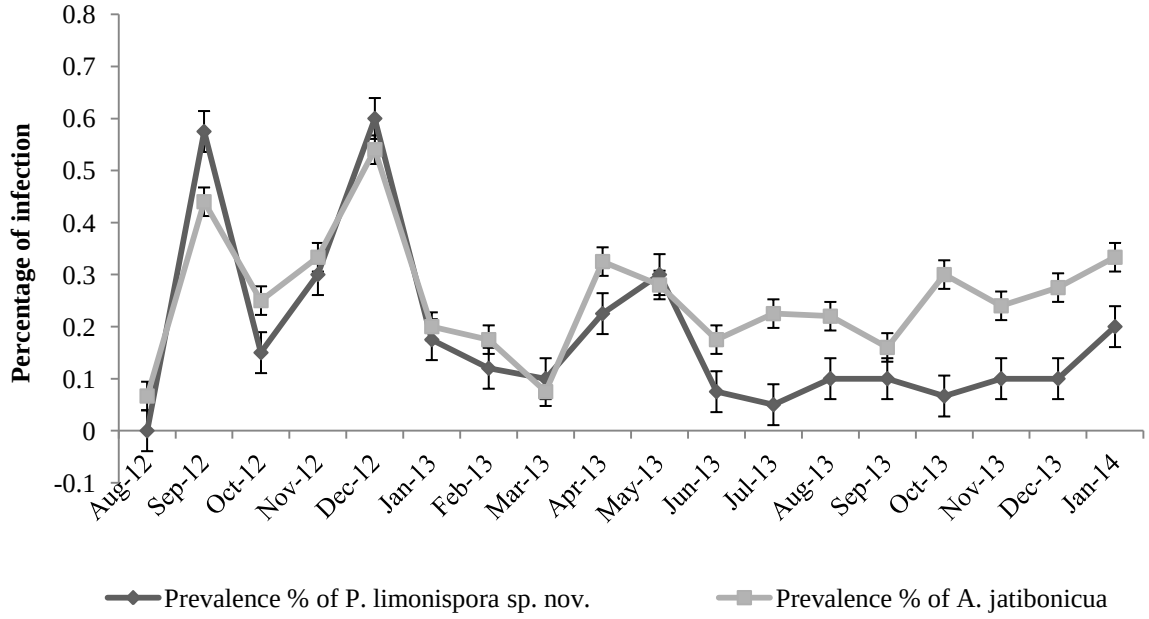

Figure 12. Prevalence of *Asellaria jatibonicua* and *Parataeniella limonispora* sp. nov. isolated from *Litthorophiloscia culebrae* over a study period of 18 months. The sampling area was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. Error bars correspond to the standard error.

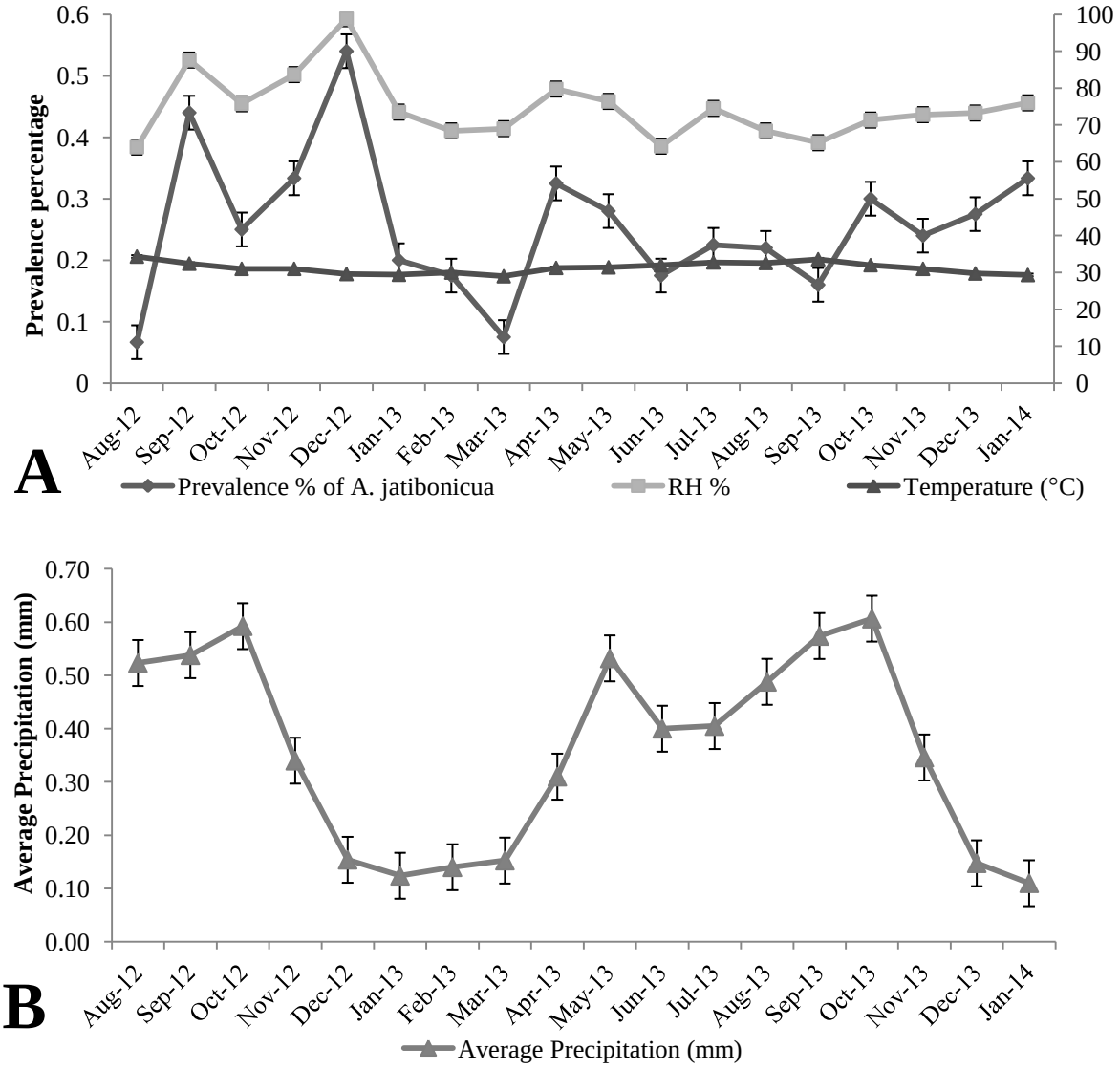

Figure 13. A- Prevalence of *Asellaria jatibonicua* isolated from *Litthorophiloscia culebrae* over a study period of 18 months compared with different environmental parameters (relative humidity and temperature). According to the repeated measures ANOVA performed there were significant differences between months (p = 0.003) and over time within months (p = 0.011). **B-** Average precipitation for the 18 months sampled. The sampling area was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. Error bars correspond to the standard error.

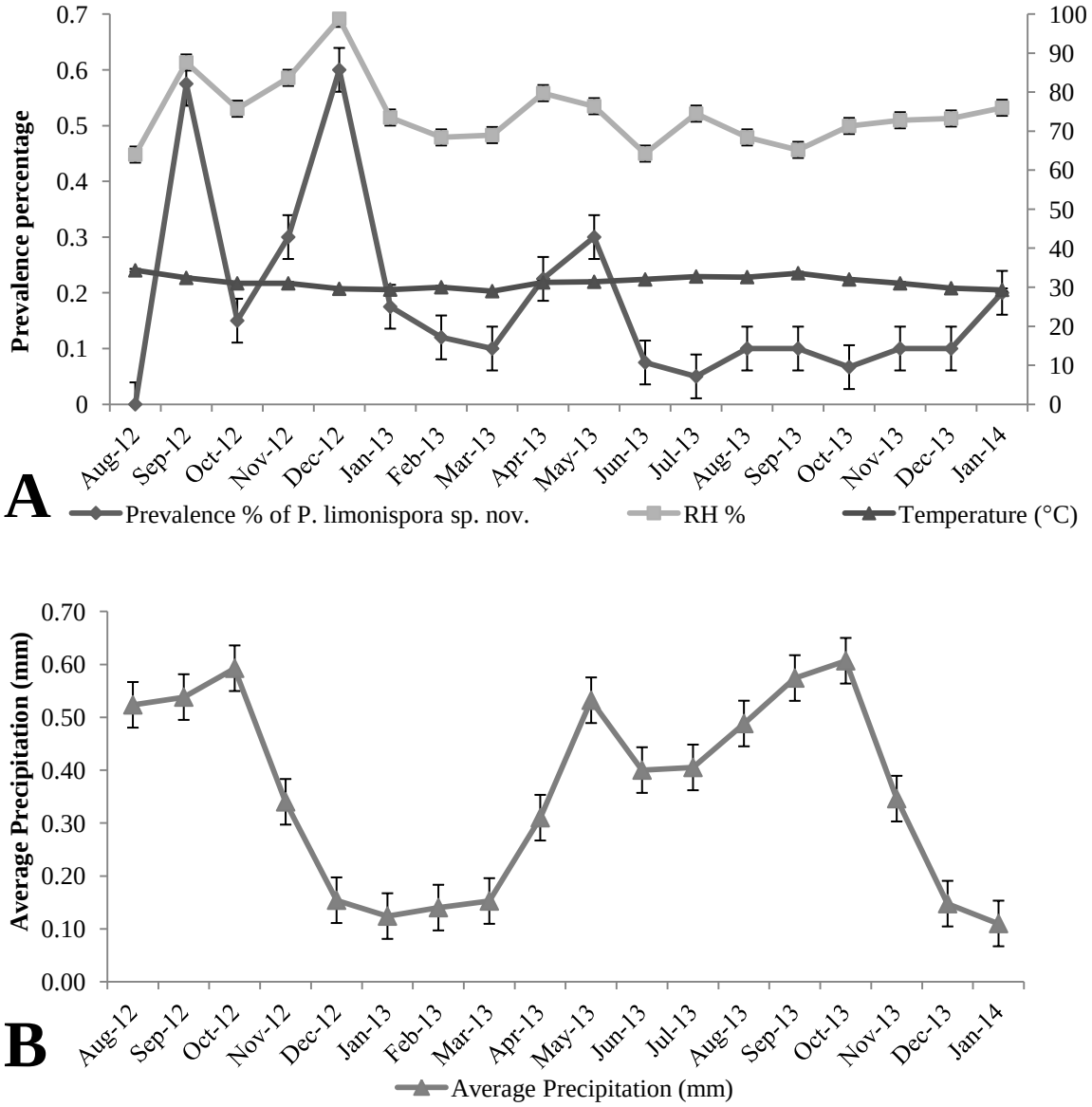

Figure 14. A- Prevalence of *Parataeniella limonispora* sp. nov. isolated from *Litthorophiloscia culebrae* over a study period of 18 months compared with different environmental parameters (relative humidity and temperature). According to the repeated measures ANOVA performed there were significant differences between months (p = 0.001) and over time within months (p = 0.004). **B-** Average precipitation for the 18 months sampled. The sampling area was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. Error bars correspond to the standard error.

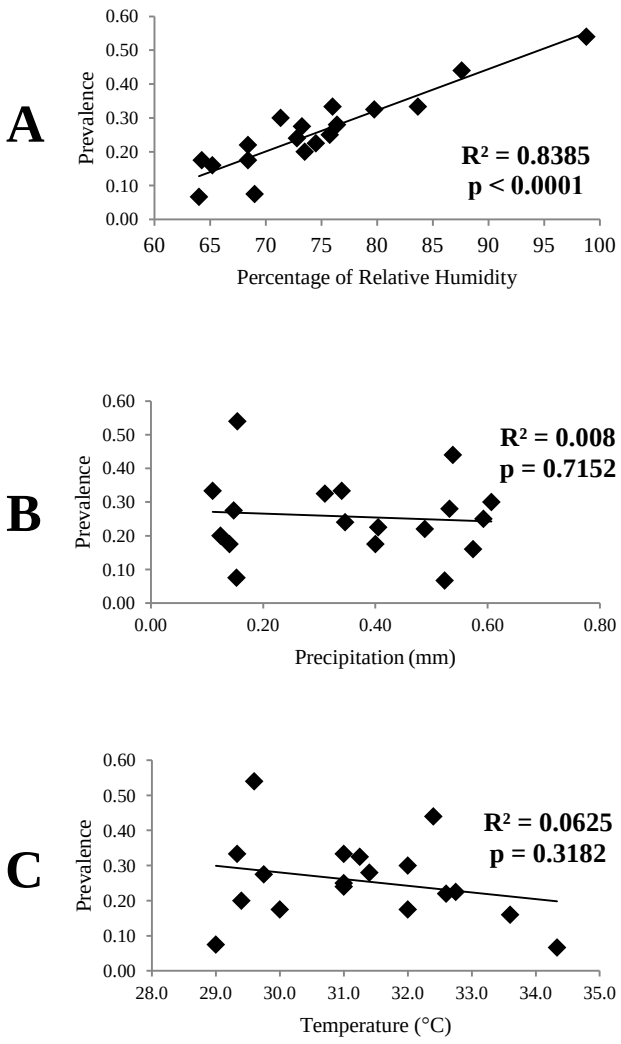

Figure 15. Linear regressions performed for the prevalence of *A. jatibonicua* (isolated from the terrestrial isopod *L. culebrae*) and environmental parameters: relative humidity (A), precipitation (B) and temperature (C).

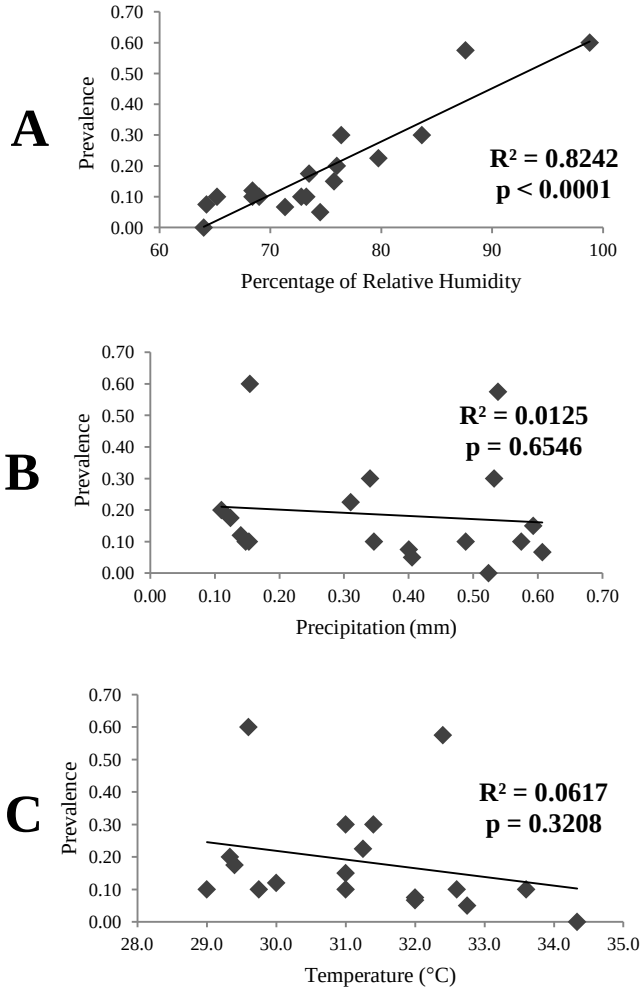

Figure 16. Linear regressions performed for the prevalence of *P. limonispora* sp. nov. (isolated from the terrestrial isopod *L. culebrae*) and environmental parameters: relative humidity (A), precipitation (B) and temperature (C).

Table 4. Results of analysis of variance for the prevalence percentage among months for the trichomycete *Asellaria jatibonicua* isolated from the terrestrial isopod *Litthorophiloscia culebrae* over a study period of 18 months. The population of isopods used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. N=75 correspond to the number of weeks sampled

Analysis of variance

Variable	N	R^2	Adj R^2	CV
% of infection	75	0.52	0.37	49.37

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	1.00	17	0.06	3.57	0.0002
Date	1.00	17	0.06	3.57	0.0002
Error	0.94	57	0.02		
Total	1.94	74			

Test: Tukey Alpha:=0.05 LSD:=0.32776

Error: 0.0165 df: 57

Date	Means	n	S.E.			
1	0.07	3	0.07	A		
8	0.08	4	0.06	A		
14	0.16	5	0.06	A	B	
11	0.18	4	0.06	A	B	
7	0.18	4	0.06	A	B	
6	0.20	5	0.06	A	B	
13	0.22	5	0.06	A	B	C
12	0.23	4	0.06	A	B	C
16	0.24	5	0.06	A	B	C
3	0.25	4	0.06	A	B	C
17	0.28	4	0.06	A	B	C
10	0.28	5	0.06	A	B	C
15	0.30	3	0.07	A	B	C
9	0.33	4	0.06	A	B	C
18	0.33	3	0.07	A	B	C
4	0.33	3	0.07	A	B	C
2	0.44	5	0.06		B	C
5	0.54	5	0.06			C

Means with a common letter are not significantly different (p > 0.05)

Table 5. Results of analysis of variance for the prevalence percentage among months for the trichomycete *Parataeniella limonispora* isolated from the terrestrial isopod *Litthorophiloscia culebrae* over a study period of 18 months. The population of isopods used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. N=75 correspond to the number of weeks sampled.

Analysis of variance

Variable	N	R^2	Adj R^2	CV
Prevalence %	75	0.57	0.44	82.63

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	1.80	17	0.11	4.40	<0.0001
Date	1.80	17	0.11	4.40	<0.0001
Error	1.38	57	0.02		
Total	3.18	74			

Test: Tukey Alpha:=0.05 LSD:=0.39672

Error: 0.0241 df: 57

Date	Means	n	S.E.			
1	0.00	3	0.09	A		
12	0.05	4	0.08	A		
15	0.07	3	0.09	A	B	
11	0.08	4	0.08	A	B	
17	0.10	4	0.08	A	B	
16	0.10	5	0.07	A	B	
13	0.10	5	0.07	A	B	
14	0.10	5	0.07	A	B	
8	0.10	4	0.08	A	B	
6	0.14	5	0.07	A	B	
7	0.15	4	0.08	A	B	
3	0.15	4	0.08	A	B	
18	0.20	3	0.09	A	B	
9	0.23	4	0.08	A	B	C
4	0.30	3	0.09	A	B	C
10	0.30	5	0.07	A	B	C
2	0.46	5	0.07		B	C
5	0.60	5	0.07			C

Means with a common letter are not significantly different (p > 0.05)

4.4. Exposure of isopods to different percentages of relative humidity.

Isopods where exposed to different percentages of relative humidity at a temperature of approximately 21^{o}C. Every isopod exposed to 100-98.2 RH % survived more than two weeks. Those that were exposed to percentages of relative humidity under 98.2 died before 16 hours of exposure. This experimental procedure was performed in three consecutive occasions and every

time the same results were obtained. Our experimental data agrees with that of Miller (1938), who stated that the optimum relative humidity for terrestrial isopods is close to 100 %. Isopods were dissected after perceiving their death. An interesting observation was that even some of the isopods that did not resisted the lower percentages of relative humidity also presented the presence of *Asellaria jatibonicua* and *Parataeniella lemonispora* sp. nov., suggesting that the isopod is not dependant of these species for their survival under stressed conditions of lower relative humidity percentages.

4.5. Fluctuations in number of isopods in population through time.

The number of isopods sampled showed a minimum of 26 in September 2014 and a maximum of 34 in January 2015 (Figure 17, Table 6). Apparently there was not a significant (Table 4) fluctuation in the number of isopods over time.

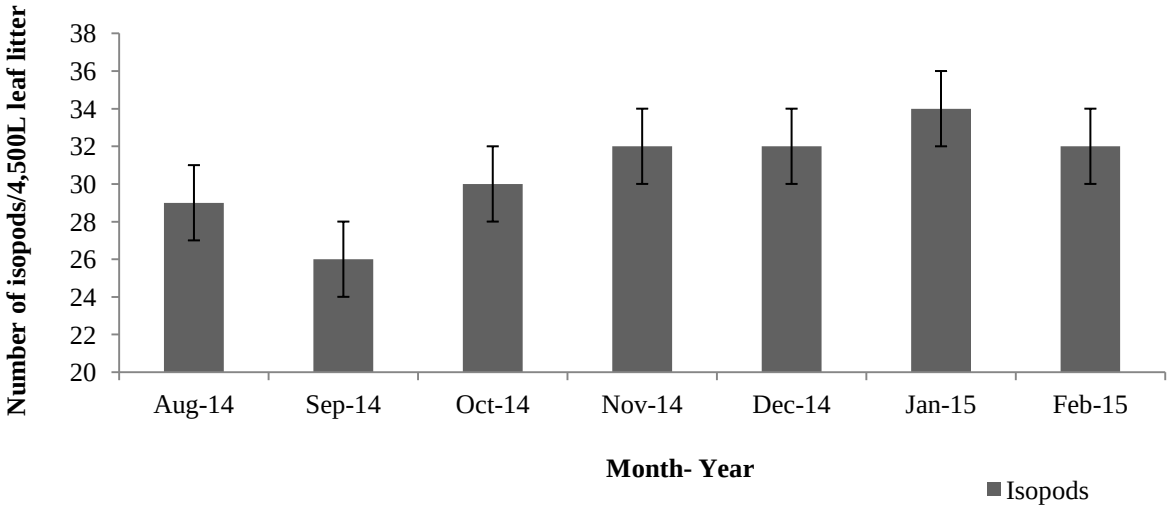

Figure 17. Fluctuation in the number of the terrestrial isopod *L. culebrae* over time. The population used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. Error bars correspond to the standard error.

Table 6. Results of analysis of variance (ANOVA) for the fluctuation in the number of isopods (*L. culebrae)* over time; N=21 correspond to the number of samples collected. The population used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus.

Analysis of variance

Variable	N	R^2	Adj R^2	CV
Isopods	21	0.06	0.00	40.55

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	1.57	6	0.26	0.14	0.9889
Month	1.57	6	0.26	0.14	0.9889
Error	26.77	14	1.91		
Total	28.34	20			

Test: Tukey Alpha:=0.05 LSD:=3.85501
Error: 1.9119 df: 14

Month	Means	n	S.E.	
Sep-14	2.87	3	0.80	A
Aug-14	3.23	3	0.80	A
Oct-14	3.33	3	0.80	A
Feb-15	3.53	3	0.80	A
Dec-14	3.57	3	0.80	A
Nov-14	3.57	3	0.80	A
Jan-15	3.77	3	0.80	A

Means with a common letter are not significantly different (p > 0.05)

Table 7. Samples of *Litthorophiloscia culebrae* collected to determine the fluctuation in the number of isopods over time. Each sample consisted of three replicates within each sample square. Each month there was a collection of nine samples. The population used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus.

Month	Sample #1	Sample #2	Sample #3	Total number of isopods sampled
Aug-14	2	1	8	**29**
	0	7	3	
	6	0	2	
	2.7	**2.7**	**4.3**	
Sep-14	8	1	0	**26**
	0	7	1	
	1	2	6	
	3	**3.3**	**2.3**	
Oct-14	4	3	2	**30**
	3	7	1	
	6	4	0	
	4.3	**4.7**	**1**	
Nov-14	0	0	9	**32**
	3	2	6	
	8	4	0	
	3.7	**2**	**5**	
Dec-14	5	2	4	**32**
	8	1	1	
	2	2	7	
	5	**1.7**	**4**	
Jan-15	7	0	1	**34**
	8	5	0	
	1	3	9	
	5.3	**2.7**	**3.3**	
Feb-15	8	1	2	**32**
	3	7	1	
	2	4	4	
	4.3	**4**	**2.3**	

Table 8. Descriptive statistics for the total number of isopod (*L. culebrae*) per 2.25 m over time; N=7 correspond to the number of months included in the sampling. The population used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus.

Variable	n	Mean	S.D.	S.E.	CV	Minimum	Maximum
Isopods	7	30.71	2.63	0.99	8.56	26.00	34.00

4.6. Isopod growth until adult stage.

Eggs inside marsupial pouches (Figure 4) were counted before offspring emersion, which ranged from 6-8 eggs per marsupium. After the new isopods were born they were measured using a stereomicroscope to determine the time of growth until the adult stage was reached. On average it took three and a half months for them to reach the average adulthood measurements. This determination was made by comparing their body measurements with those of their female progenitors. The females used in this experimental procedure ranged from 1.5-2.8 mm in length. The offspring length measurements between the first three days of their birth ranged from 0.2-0.48 mm and their length mostly depended on the number of eggs inside the marsupial pouch and body measurements of their female progenitor (Figure 18); bigger females often procreated the biggest brood (Figure 18, Appendix 1). After three and a half months the measurements of the isopods ranged from 1.2-2.6 mm.

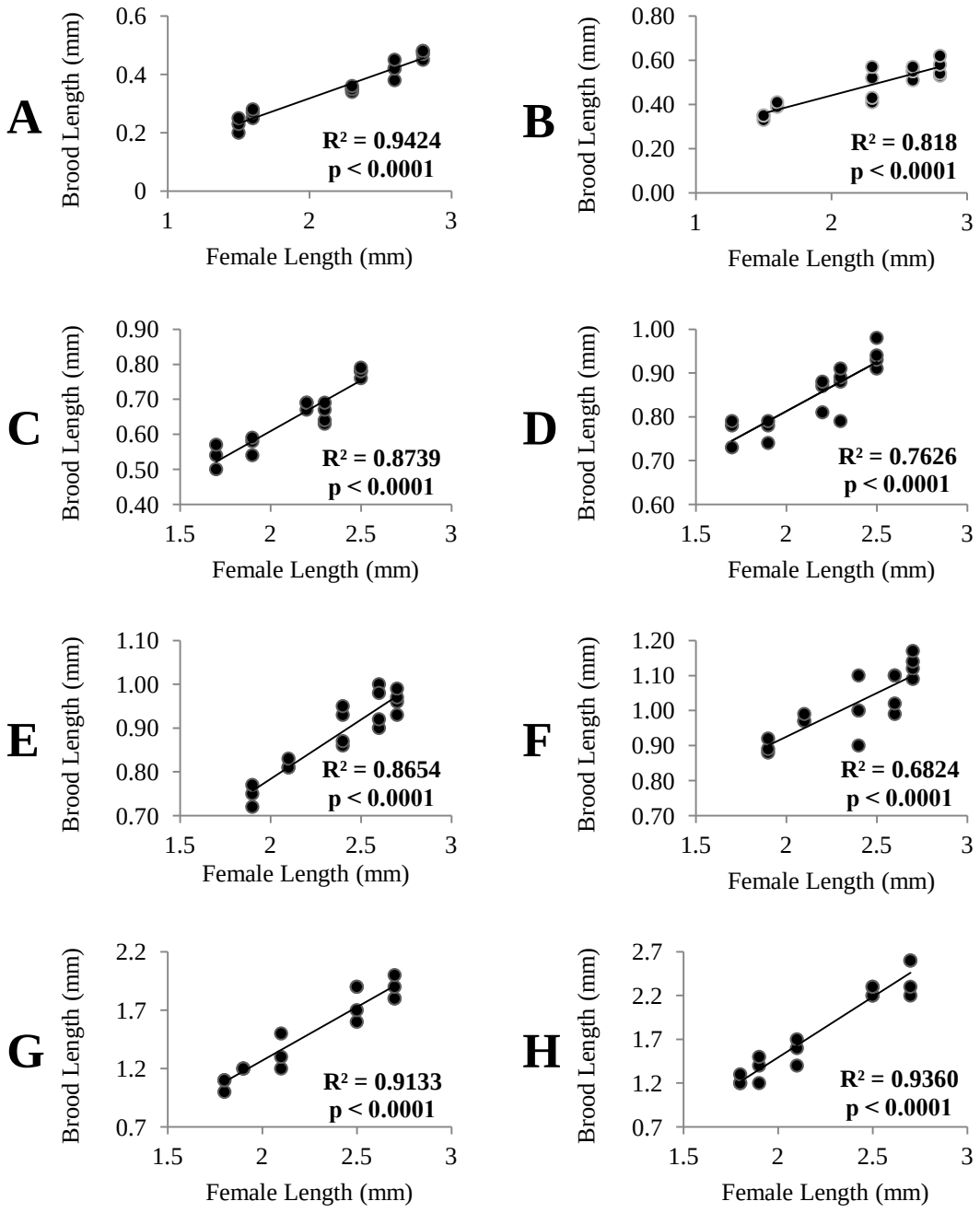

Figure 18. Linear regressions performed for the brood length of the terrestrial isopod *Litthorophiloscia culebrae* using the length of their female progenitor as the regressor. Each graph correspond to measures taken in different moments of the isopods brood development: 0-3 days (A), 2 weeks (B), 4 weeks (C), 6 weeks (D), 8 weeks (E), 10 weeks (F), 12 weeks (G) and 14 weeks (H). The sampling area for the collection of females with eggs was located in an urban area inside the University of Puerto Rico, Mayaguez Campus.

4.7. Forest sampling: Guajataca, Rio Abajo and Toro Negro

Three collections were conducted in each forest in different months of the year. On the three days selected for sampling in Toro Negro State Forest and Rio Abajo State Forest, *A. jatibonicua* was never present in the hindgut of *L. culebrae*. On the contrary the isopods sampled in Guajataca State Forest were found, at all occasions, infested with *A. jatibonicua*, with an average infestation of 40%.

Figure 19. Forests in Puerto Rico where samples of the terrestrial isopod *Litthorophiloscia culebrae* were collected, in order to evaluate the presence of *Asellaria jatibonicua* in their hindgut. G, Guajataca State Forest; RA, Río Abajo State Forest; TN, Toro Negro State Forest.

5. DISCUSSION

5.1. Prevalence and seasonality of *A. jatibonicua* and *P. limonispora* sp. nov.

There is no information available for seasonality and prevalence of trichomycetes inhabiting terrestrial arthropods. Our experimental data showed that the prevalence of both trichomycetes under study are tightly related with relative humidity (Figure 15, 16), implying a dependence of water availability for proliferation of the trichomycete; however, there was no apparent or direct relationship with temperature or precipitation. Knowing that terrestrial isopods tend to recycle their feces for nutrition purposes (Zimmer 2002), it is suggested herein that in these terrestrial habitats trichomycete spores are mainly transmitted through this type of behavior. Isopods tend to deposit their feces in the same places they eat; in this case, the vegetation debris. Furthermore, isopods, after molting, tend to consume their exuviae to regain mineral content (Hornung 2011) and thus, in this way, trichomycete spores can be reingested and dispersed as they are abundant in the lining of the hindgut, which is shed along the posterior part of the exoskeleton. Moreover, isopods spend more time sheltering from dry air and sunlight than searching for food when the climate conditions are less humid (Dias et al. 2012) and in consequence the trichomycete spores cannot be dispersed.

According to Miller (1938), the optimum relative humidity for terrestrial isopods is close to 100 %. As terrestrial crustaceans, water conservation is crucial to their survival and biology (Carefoot 1993), which makes isopods vulnerable to moisture changes and desiccation stress (Miller 1938, Edney 1954, Warburg 1987, Hassall et al. 2005, Dias et al. 2012). Their cuticle lacks an external waxy layer and is highly permeable to water (Edney 1968). Consequently, isopods depend on behavioral adaptations to prevent water loss; during high temperature and low

humid periods they tend to remain below the surface layers of leaf litter becoming inactive and they do not feed (Brody and Lawlor 1984). This behavior towards changes in humidity may explain why the results of the present study indicate a lower prevalence in the less humid months.

As in the last two decades, the environment has been changing drastically in terms of climate patterns. These changes could put in danger a large number of species around the world. Hassall et al. (2010) states that it is likely that isopods, which are at such high risk from desiccation, can detect shifts in humidity and temperature lower than 5%, since some terrestrial arthropods are able to sense and respond behaviorally to changes in humidity of 5% (Chown and Terblanche 2007). It has been shown that at lower RH and higher temperatures isopods tend to aggregate significantly more to maintain moisture and survive drought conditions (Hassall et el. 2010). Isopods being so sensitive to changes in relative humidity are also extremely vulnerable to climatic change and trichomycetes as well, since they depend on their hosts for the continuity of their life cycle, for they are known to be strict symbionts (they cannot live outside the gut of their host). Trichomycetes spores can be dispersed from one place to another in the isopods' feces present in the leaf litter, but cannot emerge and develop if they are not present inside the gut of its symbiont. In consequence, if isopods disappear from a population the trichomycetes will do as well.

5.2. Seasonality and prevalence: aquatic environment versus terrestrial.

All inquiries that have been made that deal with seasonality and prevalence of trichomycetes are related to aquatic hosts; this being the first investigation made in a terrestrial environment. In this study, experimental data showed a variation of percentages of infection

throughout the year (Table 4, 5). The highest peaks were in September 2012 and December 2012, which agrees with the most humid months of the study period. In fact, statistical measurements (Figure 15, 16) confirmed a linear regression for the prevalence of the two trichomycetes under study with changes in relative humidity in contrast to temperature and precipitation. Beard and Adler (2002) also reported statistical differences between seasons, but for the occurrences of trichomycetes inhabiting black flies (Diptera: Simuliidae). They recorded a variation by season of the prevalence of *Harpella melusinae* but not by year, with a higher prevalence in summer and fall. They also reported infections of 100% for the same trichomycete over the entire sampling year. In seasonality studies by Siri et al. (2008) on trichomycetes (Harpellales) from chironomid larvae present in phytotelmata, the authors reported that even though there was not a statistically significant difference in prevalence of trichomycetes, the infection was not constant over the year, having a higher prevalence during spring, summer and fall with the exception of spring 2003 and fall 2004 that had a low prevalence of trichomycetes. In the same study, they compared the prevalence with environmental variation in temperature, humidity and rainfall and stated that none of the variables affected the prevalence of the Harpellales; however, they hypothesized that transmission occurs easily in the warmer months. In another seasonality study by Reeves (2004), he published data that showed a significant variation of Harpellales-infected mosquito larvae throughout the year. He hypothesized that this variation could depend on environmental factors or the life cycle of the larval mosquitoes, since the months of highest infection corresponded to those at the beginning (March) and end (October) of the feeding period of the larvae. We can compare Reeves assumption with ours, since earlier on this manuscript we proposed the idea of a tight dependence of the trichomycete on the behavior of its host. Our data showed a highest prevalence in those periods (most humid)

when the isopods are more likely to be on the surface layers of the leaf litter, where they are active and constantly feeding (as Reeves' assumption). We also mentioned that when the relative humidity lowers down the isopods tend to spend more time sheltering, inactivate and do not feed (this is the time when trichomycetes cannot be dispersed or ingested to continue their life cycle).

5.3. Morphological aspects of *Parataeniella limonispora* sp. nov.

There are only six species described for the genus *Parataeniella*, which have been found inhabiting the hindgut of terrestrial isopods. In comparison to the other species of *Parataeniella* the newly described species in this study is short in length (up to 150 µm long), possesses only uniseriated and uninucleated thin-walled lemoniform sporangiospores. The sporangiospores of *P. limonispora* sp. nov. are released through an apical aperture in the thallus resembling *P. dilatata*, but the latter species' thallus is much larger (100-225 µm long by 12-14 µm wide) than that of our species. *Parataeniella scotonisci* (up to 80 µm long by 3.5-9 µm wide) is the closest species to *P. limonispora* sp. nov. (up to 150 µm long by 6-9 µm wide) in thallus dimensions and sporangiospore morphology (oval to lemon shape form), although it possesses both, primary and secondary binucleated sporangiospores that can be uni- or biseriated, which have not been observed in the new species. According to Lichtwardt et al. (2001), the other six species of *Parataeniella* include primary and secondary sporangiospores at some point in their life cycle, the latter have not been observed in *P. limonispora* sp. nov. Furthermore, most other species of *Parataeniella* produce their sporangiospores in an uni- or biseriated form, but only uniseriated arrangement was seen in the new species. The thallus is always unbranched and sporangiospores are released through an apical opening instead of a longitudinal slit of the thallus as in the case of *P. armadillidii*.

This newly discovered species was present in two localities of Puerto Rico (University of Puerto Rico, Mayaguez Campus and Guajataca State Forest) and in the same populations of isopods in which *Asellaria jatibonicua* was also present. As stated earlier in the text, the trichomycete's prevalence data collected from the population inside the University of Puerto Rico, Mayaguez Campus showed a tight relationship with the host's sensitivity to relative humidity. This fact opened many questions: if there is really a dependence of high relative humidity for the development of the trichomycetes inside its host; then, why we did not find any trichomycetes in the humid forests Rio Abajo State Forest and Toro Negro State Forest? This discovery also open the question if both trichomycetes behaved almost the same way; then, every trichomycete living inside terrestrial hosts will behave the same way? Is it just relative humidity? or is there another factor as the type of food (ea. specific tree leaves, nutrients in soil, etc.) consumed by the host that makes more probable the development and survival of the trichomycetes spores? For instance, our trichomycetes-infected population of isopods under study is located under a *Calophyllum brasiliense* tree. These are very interesting questions that should be answered and explored in further experiments and investigations.

5.4. Are trichomycetes really commensals?

According to Eberl (2010), many eukaryotes have overcome their nutritional limitations (e.g. often when their diet consist of food either limited in essential amino acids and vitamins, or difficult to digest) by associating with microorganisms. In addition, he states that these symbiotic associations can be important drivers of evolution. Isopods are considered to be the perfect model to study the changes in microbe gut community composition between related taxa living in different environments (Eberl 2010), since they can be found in different types of habitats

ranging from strictly marine to strictly terrestrial (Abbott 1939; Edney 1968; Broly et al. 2013). There is evidence of symbiotic bacteria living in the hepatopancreas of terrestrial isopods associated with cellulose digestion and phenol oxidation (Zimmer and Topp 1998; Zimmer et al. 2002). In addition, these bacteria have shown to increase the survival of its host in conditions of low quality diets (Fraune and Zimmer 2008). As well, some fungi are known to produce enzymes capable of digesting lignin, cellulose and other polymers in the gut of arthropods that are resistant to degradation by their own enzymes (Breznak and Brune 1994; Douglas 1994; Zimmer et al. 2001; Zimmer et al. 2002).

Often it is said that trichomycetes share a commensalistic association with their hosts (Lichtwardt et al. 2001). Although, there is evidence of various cultivable species that have shown to impart benefits to their hosts and others to be detrimental (Horn and Lichtwardt 1981; Sweeney 1981; Labeyrie et al. 1996). Why do we say then that our trichomycetes are merely commensals? Trichomycetes may impart benefits to their hosts by helping them to digest difficult components of the leaf litter (such as lignin, cellulose, and other polymers) or by producing nutrients that are deficient in the isopod's diet. Moreover, if isopods that contain these microorganisms are healthier (in a nutritional matter), may be more capable of surviving adverse environmental condition or predation than other isopods that do not possess this advantage. Also, this association could provide isopods a better reproduction, a larger number of eggs, and optimal body length. These hypotheses are difficult to test because the system is hard to manipulate. Trichomycetes are not cultivable and hosts need to be killed in order to assess their presence. Hence, testing these ideas need new ways to look at isopods in the laboratory or developed population with no infection.

6. FUTURE PROJECTS

6.1. Food quality and terrestrial isopods

The influence of food quality on the population dynamics of herbivores has been intensively studied, and have shown to be important on limiting their population dynamics (Lawton and McNeil 1979; Crawley 1983; Dempster 1983). However, we know very little about decomposers (Rushton and Hassall 1987). Rushton and Hassall (1987) states that since most decomposers have a wide range of diet and dead organic matter (relatively abundant in many ecosystems), it is often assumed that detrivores are surrounded by an abundance of suitable food. In another comprehensive review, Warburg et al. (1984) suggest that food is not important on influencing the population fluctuations of terrestrial isopods, and alternatively they propose that it is controlled by climatic changes in temperature and humidity, since these strongly affect growth, reproduction, and survivorship of isopods. However, other studies have demonstrated that food quality influence growth and survivorship of isopods (Cromack 1967; Merriam 1971). Furthermore, investigations with grasslands isopods have shown that freshly fallen leaves from dicotyledonous plants provides them the best quality food (shown by their growth and survivorship) (Rushton and Hassall 1983). These findings arouse in us the questions of what kind of food does the terrestrial isopod *L. culebrae* prefer or is best for their development and if their trichomycetes symbionts have any role on the assimilation of nutrients in nutrient deficient environments. We did not investigate these issues, but found the presence of the isopod under study in different areas of Puerto Rico that hold different kinds of leaf litter composition and did not found the presence of its trichomycetes in all the locations. In consequence, we hypothesize

that trichomycetes may have a role on the isopods utilization of different kinds of leaf litter difficult to digest. These might be the theme of another investigation.

6.2. Climate change and isopods feeding

Climate change and in particular an increase in temperature could have a direct impact in the moisture of soil (Römbke et al. 2011). Not much is known of the effect global warming could have on individual species. Terrestrial isopods in particular, being small organisms, abundant, easy to handle, and dependant on suitable soil moisture levels (Drobne 1997, Hornung et al. 1998) represents a good model for experiments regarding climatic change. In future projects with the species of terrestrial isopods we studied (L. culebrae) should be measured the effect of temperature and soil moisture apart, and the relationship these two have together on the behavior of the isopods.

According to Römbke et al. (2011), species from the Mediterranean region are adapted to food from Mediterranean plants (with differences in nutrient status and structural characteristics), higher temperatures and lower soil moisture levels, indicating that their feeding rate could be lower if they obtain their food resources from temperate plants and if they live at lower temperatures. They also hypothesize that for temperate species the opposite could be true. In other words, if these organisms do not adapt to these climatic changes this could lead to lower feeding rates and thus lower rates of decomposition of organic matter. In addition, to the extinction of its trichomycetes symbionts, since they strictly depend to their hosts feeding behavior.

7. CONCLUSIONS

In this study we have presented the first ecological data of trichomycetes inhabiting a terrestrial host. Additionally, we have shown important information about the ecology of the terrestrial isopod *Litthorophiloscia culebrae* (host).

1. The prevalence of *Asellaria jatibonicua* and *Parataeniella limonispora* is tightly dependant to the biology of *Litthorophiloscia culebrae*, since both trichomycetes showed higher prevalence in the most humid months and lower prevalence in the less humid months.

2. There is no direct relationship between the prevalence of trichomycetes with temperature and precipitation.

3. *Litthorophiloscia culebrae* possess a minimum relative humidity (RH) resistance of 98.2 percentage and an optimum survival at 100 percentage RH in laboratory controlled RH.

4. The trichomycetes under study do not influence the survival of the isopods during the period of controlled relative humidity under laboratory conditions. Since even some of the isopods who did not resists relative humidities below 98.2 percentage had the presence of both trichomycetes under study. Additionally, the trichomycetes were present in some of the isopods placed under relative humidities of 100-98.2 percentage.

5. *Litthorophiloscia culebrae* has an average growth time of three and a half months to reach adult stage. The size of the isopods when born depend on that of their female progenitor. Larger females often procreate larger brood. In addition, each female can carry 6-8 eggs inside its marsupial pouch.

6. The fluctuation in number of isopods in the population over time was not statistically significant. For this reason we can conclude that the number of isopods in that population is almost constant over time.

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APPENDICES

Appendix 1. Tables of the isopods' (*L. culebrae)* offspring measurements for determination of growth time until adult stage. The sampling area for the collection of females with eggs was located in an urban area inside the University of Puerto Rico, Mayaguez Campus.

Petri dish #	Female Length	# of eggs	Brood Length (0-3 days)	Brood Length (2 weeks)
1	1.5	6	0.2	0.3
			0.23	0.4
			0.25	0.4
2	1.6	6	0.25	0.4
			0.27	0.4
			0.28	0.4
3	2.3	8	0.34	0.4
			0.35	0.4
			0.35	0.5
			0.36	0.6
4	2.8	8	0.45	0.5
			0.47	0.5
			0.48	0.6
			0.48	0.6
5	2.6	8	0.38	0.5
			0.42	0.6
			0.42	0.6
			0.45	0.6

Petri dish #	Female Length	# of eggs	Brood Length (4 weeks)	Brood Length (6 weeks)
6	2.2	6	0.7	0.8
			0.7	0.9
			0.7	0.9
7	1.9	6	0.5	0.7
			0.6	0.8
			0.6	0.8
8	2.5	8	0.8	0.9
			0.8	0.9
			0.8	0.9
			0.8	1.0
9	1.7	6	0.5	0.7
			0.5	0.8
			0.6	0.8
10	2.3	8	0.6	0.8
			0.6	0.9
			0.7	0.9
			0.7	0.9

Petri dish #	Female Length	# of eggs	Brood Length (8 weeks)	Brood Length (10 weeks)
11	2.7	8	0.9	1.1
			1.0	1.1
			1.0	1.1
			1.0	1.2
12	2.1	6	0.8	1.0
			0.8	1.0
			0.8	1.0
13	1.9	6	0.7	0.9
			0.8	0.9
			0.8	0.9
14	2.6	8	0.9	1.0
			0.9	1.0
			1.0	1.1
			1.0	1.1
15	2.4	8	0.9	0.9
			0.9	1.0
			0.9	1.0
			1.0	1.1

Petri dish #	Female Length	# of eggs	Brood Length (12 weeks)	Brood Length (14 weeks)
16	2.7	8	1.8	2.2
			1.8	2.3
			1.9	2.6
			2.0	2.6
17	2.5	8	1.6	2.2
			1.7	2.2
			1.9	2.3
			1.9	2.3
18	1.8	6	1.0	1.2
			1.1	1.2
			1.1	1.3
19	1.9	6	1.2	1.2
			1.2	1.4
			1.2	1.5
20	2.1	6	1.2	1.4
			1.3	1.6
			1.5	1.7

Appendix 2. Comparison of species of *Parataeniella* Poison with the newly discovered species (*P. limonispora*) from Puerto Rico. All the descriptive information that correspond to the other species of *Parataeniella* were directly extracted from Lichtwardt et al. (2001).

Name	Thalli long	Thalli wide	Sporangiospores	
			Secondary	Primary
P. armadillidii	Up to 385 µm	12-15 µm	~ 12 x 10 µm, binucleate	Uninucleate, globose to angular in shape, ~11 µm diameter, biseriately, rarely uniseriately, in a saccate thallus, emerging from thallus through a longitudinal slit.
P. dilata	Thalli up to 300 µm or 100-225 µm	12-14 µm or 22-28 µm	2- (to 5-) nucleate secondary infestation sporangiospores 45-50 µm long.	Uninucleate sporangiospores 15-35 µm long or uniseriate or biseriate thick-walled, oval to ellipsoidal, or uni- or binucleate primary infestation sporangiospores 16-22 x 9-11 µm, emerging from an apical orifice in the thallus.
P. flavospora	Mature thalli 210-690 x 6-16 µm	Up to 21 µm	Binucleate, thin-walled, (18-)22(-39) x 6-9 µm.	Binucleate, thick-walled, oval to ellipsoidal, uniseriate within sporangium. Mature spores consistently full of lipid bodies, yellowish, 15-24 x 7-12 µm.
P. latrobi	Short to more than 2 mm long		Thallus dividing to form a row of sporangia, each producing one binucleate secondary infestation sporangiospores measuring 40-70 x 15-19 µm.	Producing within the thallus a uniseriate or biseriate row of uninucleate, oval to angular, primary infestation sporangiospores measuring (25-)31-35 x 15-18 µm.
P. mercieri	170-180 µm	10-12 µm	2- to 4-nucleate, 20-25(-35) µm long, or uninucleate and more or less isodiametric, 10-12 µm.	Uninucleate, ellipsoidal, uniseriate within the thallus, 25-30 x 10-12 µm. Thalli may produce series of secondary infestation sporangia terminally and a primary infestation sporangium basally in the same thallus. Type species.
P. scotonisci	Up to 80 µm	3.5-9 µm	Uni- or binucleate	11-14.5 X 2.5-4.5 µm Binucleate, uni- or biseriate.
P. lemonisporum	Up to 75 µm	6-9 µm	None	12-15 X 6-9 µm, uninucleate, 4 to 8 sporangiospores, uniseriate.

Appendix 3. Mean ± SD of the prevalence of *Asellaria jatibonicua* and *Parataeniella limonispora* measured over a study period of 18 months. The population of isopods used for this study was located in an urban area inside the University of Puerto Rico, Mayaguez Campus. The *p* values are the result of repeated measures ANOVA.

Prevalence	Mean ± SD	*p* value	
		Between months	Over time within months
Asellaria jatibonicua		0.003	0.011
Aug-12	0.07 ± 0.12		
Sep-12	0.44 ± 0.13		
Oct-12	0.25 ± 0.06		
Nov-12	0.33 ± 0.06		
Dec-12	0.54 ± 0.05		
Jan-13	0.20 ± 0.12		
Feb-13	0.18 ± 0.10		
Mar-13	0.08 ± 0.10		
Apr-13	0.33 ± 0.10		
May-13	0.28 ± 0.22		
Jun-13	0.18 ± 0.17		
Jul-13	0.23 ± 0.17		
Aug-13	0.22 ± 0.08		
Sep-13	0.16 ± 0.11		
Oct-13	0.30 ± 0.10		
Nov-13	0.24 ± 0.11		
Dec-13	0.28 ± 0.13		
Jan-13	0.33 ± 0.23		
Parataeniella limonispora		0.001	0.004
Aug-12	0.00 ± 0.00		
Sep-12	0.46 ± 0.34		
Oct-12	0.15 ± 0.10		
Nov-12	0.30 ± 0.10		
Dec-12	0.60 ± 0.10		
Jan-13	0.14 ± 0.22		
Feb-13	0.15 ± 0.13		
Mar-13	0.10 ± 0.08		
Apr-13	0.23 ± 0.15		
May-13	0.30 ± 0.24		
Jun-13	0.08 ± 0.10		
Jul-13	0.05 ± 0.10		
Aug-13	0.10 ± 0.10		
Sep-13	0.10 ± 0.07		
Oct-13	0.07 ± 0.06		
Nov-13	0.10 ± 0.10		
Dec-13	0.10 ± 0.08		
Jan-13	0.20 ± 0.20		