

Diversity of nematodes associated with *Heterodermia diademata*: A case study in the Eco Área of Avellaneda, Buenos Aires, Argentina

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ABSTRACT. Lichens represent a complex life form characterized by a symbiotic partnership between at least two organisms: a fungus and an alga. In this unique relationship, the fungus offers a protective habitat for the alga, which, in turn, harnesses energy from sunshine, water and air to provide sustenance for the fungus. They fulfill ecological roles as a food source for many invertebrates and serve as the basal trophic level for a variety of animals, being considered micro-ecosystems which can host a rich and diverse biota. Nematodes are among the most widespread and abundant of all metazoans, inhabit moist interstitial environments in all habitats and are abundant in marine and freshwater benthic habitats and in the soil. They were also reported from accumulations of detritus in leaf axils, in the angles of tree branches and in mosses and lichens. In this case study, the trophic role and diversity of free-living nematodes associated with lichens were examined in an area of Buenos Aires province, Argentina, revealing seven genera of nematodes linked to *Heterodermia diademata*, representing all trophic levels of free-living nematodes: bacteriophages (*Plectus* and *Acrobeles*), fungivores (*Aphelenchus*), herbivores (*Helicotylenchus*), omnivores (*Eudorylaimus* and *Mesodorylaimus*) and a predator (*Prionchulus*).

[Keywords: lichen, ecosystem role, fungivorous, Buenos Aires]

RESUMEN. Diversidad de nematodos asociados a *Heterodermia diademata*: Un estudio de caso en la Eco Área de Avellaneda, Argentina. Los líquenes representan una forma de vida compleja caracterizada por una asociación simbiótica entre al menos dos organismos: un hongo y un alga. En esta relación única, el hongo ofrece un hábitat protector para el alga, la que, a su vez, aprovecha la energía de la luz solar, el agua y el aire para proporcionar sustento al hongo. Cumplen roles ecológicos como fuente de alimento para muchos invertebrados y sirven como nivel trófico basal para una variedad de animales; se los considera microecosistemas que pueden albergar una biota rica y diversa. Los nematodos se encuentran entre los metazoos más extendidos y abundantes, habitan ambientes intersticiales húmedos en todos los hábitats y son abundantes en hábitats bentónicos marinos y de agua dulce y en el suelo. También se los encontró en acumulaciones de detritos en las axilas de las hojas, en los ángulos de las ramas de los árboles y en musgos y líquenes. En este estudio de caso se examinó el papel trófico y la diversidad de nematodos de vida libre asociados con líquenes en un área de la provincia de Buenos Aires, Argentina. Se revelaron siete géneros de nematodos vinculados a *Heterodermia diademata*, que representan todos los niveles tróficos de nematodos de vida libre: bacteriófagos (*Plectus* y *Acrobeles*), fungívoros (*Aphelenchus*), herbívoros (*Helicotylenchus*), omnívoros (*Eudorylaimus* y *Mesodorylaimus*) y un depredador (*Prionchulus*).

[Palabras clave: líquen, rol ecosistémico, fungívoro, Buenos Aires]

INTRODUCTION

Lichens are a complex life form that can be defined as a self-sustaining ecosystem formed by the interaction of a fungal exhabitant and an extracellular arrangement of one or more photosynthetic partners, along with an indeterminate number of other microscopic organisms (Nash 2008; Hawksworth and Grube 2020). They are found in almost any type of ecosystem, can tolerate the most extreme environments on Earth –from hot deserts to arctic regions– and are characterized by low growth rates that enable them to become pioneer organisms in the colonization of fresh rocks and in soil formation (Chapin 1980; Raggio et al. 2011; de Vera 2012; Kaasalainen et al. 2017). Lichens also play an important role in nutrient cycling and serve as the basal trophic level for a variety of animals, including nematodes (Cornelissen et al. 2007; Asplund and Wardle 2017). Studies showed lichen species differ greatly in nutrient concentrations and carbon-based secondary compounds (Asplund and Wardle 2013, 2017), which can, in turn, influence both the consumption and decomposition of lichen material by associated invertebrates (Gauslaa 2005; Pöykkö et al. 2005). They also have a particular sensitivity to capture moisture and absorb heavy metals from the environment, for which they have been studied as bio-indicators (Weldon and Grandin 2021; Belguidoum et al. 2022).

Nematodes are among the most widespread and abundant of all metazoans. These worms inhabit moist interstitial environments in all habitats and are abundant in marine and freshwater benthic habitats, in the soil and as parasites in a great variety of plant and animal hosts. They have also been reported from accumulations of detritus in leaf axils, in the angles of tree branches and in mosses and lichens (Rupert et al. 2004). Due to the diversity of their trophic groups, nematodes play a key role in the environment participating in nutrient cycling and in the regulation of organisms in the food web through predator-prey relationships and through their close relationship with plant hosts, as well as with mosses and lichens (Zheng et al. 2022). Because of their sensitivity to environmental disturbances, nematodes have also been studied as environmental bioindicators through indices such as the maturity index, the enrichment index and the trophic web structure index (Salas and Achinelly 2020; Young and Unc 2023). Associations have

been described between these two potential bioindicators, in which lichens harbor various species of nematodes and provide shelter and food for survival even in extreme conditions (Caldwell 1981a,b; Siddiqi and Hawksworth 1982; Pickup 1990; Darby et al. 2007; Liu et al. 2011; Bokhorst et al. 2015; Asplund and Wardle 2017). To our knowledge, there are no studies on such associations of these two environmentally important organisms in Argentina. This case study provides new information on nematode micro-fauna diversity in the association of these two organisms of ecological importance in a region of Buenos Aires province, Argentina.

MATERIALS AND METHODS

Lichen samples were collected during 2021 in the Eco Área of Avellaneda (45 ha), Buenos Aires, Argentina (34°39'51.7" S-58°18'56.3" W) (Figure 1). This is an urban municipal reserve (belonging to the Avellaneda municipality) that conserves around 300 species of wild fauna and flora, and ecosystems like wetland and secondary riparian forest. Six different locations of the Eco Área were chosen based on the presence of abundant foliose lichen, presumably of the same species. The locations have no apparent vegetation differences. The survey was carried out by ocular inspection on tree logs and lichen samples were removed without stripping the bark with a metal blade. Two samples were taken from each tree and collected on *Salix* sp. The distance among the trees ranged approximately between 15 and 20 m, and on the same tree, the lichens were distanced by 5 to 20 cm. The material was taken to the laboratory in paper bags and stored in a refrigerator at 4 °C for further analyses.

Lichen processing and identification

Samples (two per tree) were processed using a scalpel to separate and open the thallus under a NIKON SMZ800N stereomicroscope. For their identification, observations of the morphological and anatomical characteristics were made with an optical microscope (Arcano Ztx). Spot color tests were also performed in the thallus with reagents C (NaClO) and K (KOH) (Brodo et al. 2001).

Nematode processing and identification

For nematode isolation, 100 cm³ of previously triturated lichen thallus were taken from each sample and centrifuged for 3 minutes at 1210

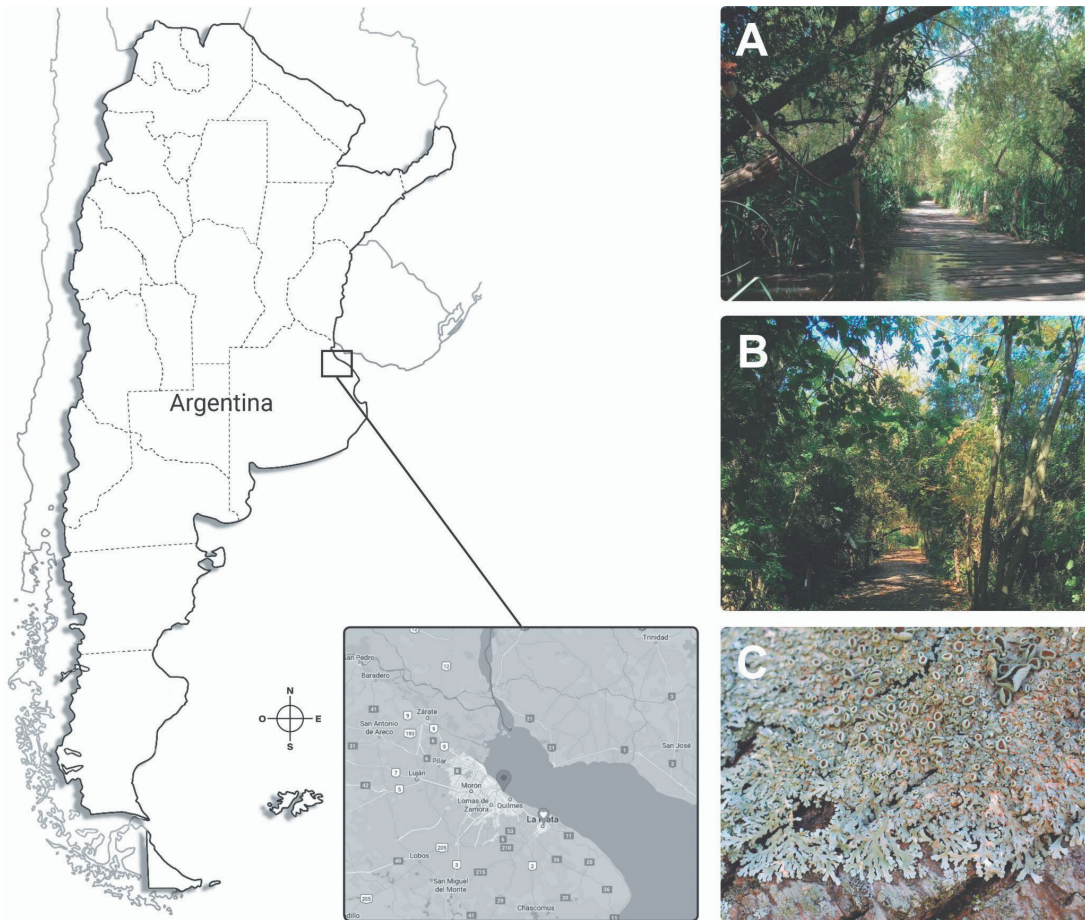


Figure 1. Location of the Eco Área de Avellaneda, Buenos Aires province, Argentina. A and B) Trails of the reserve, where the specimens of lichens were collected. C) Specimen of *Heterodermia diademata*.

Figura 1. Ubicación de la Eco Área de Avellaneda, provincia de Buenos Aires, Argentina. A y B) Senderos de la reserva, donde se recogieron los ejemplares de líquenes. C) Ejemplar de *Heterodermia diademata*.

g (Macro Centrífuga Giumelli® Z-29) to eliminate unwanted material. The resulting supernatant containing residual organic matter was discarded. The decanted material was homogenized again in a sucrose solution (484 g sucrose/L H₂O) and centrifuged for 3 minutes at 1210 g (Caveness and Jensen 1955 modified). The supernatant with nematodes in suspension was poured into screens with a 40- μ m opening, collecting and depositing the specimens in a test tube with Ringer's solution. This was followed by incubation in a water bath at 60 °C for 2 minutes to instantaneously heat-kill the nematodes, and subsequent fixation in T.A.F. (2% triethanolamine, 7.5% formaldehyde in distilled water) (Hazir et al. 2022). The specimens were mounted on permanent glass slides for morphometrical analysis, measured with an ocular micrometer in a Leica DM 500 microscope

and determined at genus level after the keys described in Manzanilla-López and Marbán-Mendoza (2012) and Chaves et al. (2019). All measurements are in micrometers unless otherwise specified and are presented as the range followed by the mean in parentheses.

Nematode ecology

According to the structure and diversity of the nematode community, the specimens were analyzed in order to obtain an ecological inference of these organisms in association with lichen samples. The determined nematodes were placed in the categories proposed by Yeates et al. (1993): bacterivorous, fungivorous, plant feeder, omnivore and predator. Nematodes can behave as colonizing or persistent organisms, reflecting changes in the soil in which they inhabit. Based on

these characteristics, Bongers (1990) assigned families of nematode values of 1-5 on a colonizer persister (cp) (the scale is named class pp when referring to plant-feeder nematodes). The cp 1 nematodes have short life cycles and are tolerant to environmental disturbances, being considered colonizers and dominant in soil samples with anthropic intervention. The cp 2 nematodes have longer life cycles and less fertility than cp 1; they are very tolerant against polluting conditions, indicating polluted soils. The cp 3, cp 4, and cp 5 nematodes have even longer life cycles and are considered persistent nematodes, indicating less disturbed soils (Salas and Achinelly 2020).

Maturity indices were determined using the NINJA (Nematode INDicator Joint Analysis) software (Sieriebriennikov et al. 2014). The maturity index (MI) represents a semiquantitative value based on the ability to colonize environments (cp value) by free-living nematodes. A low MI value will indicate greater disturbance in the environment, and high values indicate stable environments (Bongers 1990). This index is calculated by

$$MI = \sum (vi \times fi) / n \quad \text{Equation 1}$$

where vi =colonizer-persister value (cp) (assigned to the family), fi =frequency of family i in the sample, and n =total number of individuals in a sample).

It is known that nematodes respond differentially (even in small patches of substrate) to enrichment events or structural disturbances, which can be reflected by the enrichment index (EI) and structure index (SI) (Ferris and Bongers 2006). These indices consider the presence or absence of certain nematode trophic groups, allowing the construction of a profile of the nematode fauna present in the ecosystem and reflecting whether the nematode community is in an enriched or structured state. (Neher 2001; Neher and Darby 2009; Salas and Achinelly 2020). High values of the Enrichment index are given by the prevalence of primary consumers (bacterivorous and fungivorous nematodes), which may indicate events where the soil or substrate has a high intake of organic matter or nutrients. High values of the structure index indicate stable trophic webs, with a high prevalence of predatory and omnivorous nematodes that control the growth of nematode populations at lower levels of the food web (Talavera et al. 2021).

RESULTS

The lichen samples were identified as *Heterodermia diademata*, which is a corticolous species that performs symbiosis with green algae. It is mostly found on tree trunks and branches. It is distributed worldwide in tropical and subtropical areas; in Argentina, it is a common species that can be found from the center to the north of the country. During the study of the nematode diversity associated with *H. diademata* in the Eco Área of Avellaneda, a total of 86 nematodes (*Acrobeles* sp. [n=1], *Plectus* sp. [n=13], *Aphelenchus* sp. [n=1], *Helicotylenchus* [n=1], *Eudorylaimus* sp. [n=19], *Mesodorylaimus* sp. [n=23] and *Prionchulus* sp. [n=28]) were found.

The nematodes of the genus *Acrobeles* were characterized by a lip region with triangular lips and bifurcated labial probollae, bordered by triangular tines. The esophageal corpus was cylindrical, with a terminal valved bulb, the cuticle striated and the tail short.

The bacterivorous *Plectus* was characterized by a lip region separated from the body by a constriction and a thick and short cephalic sensilla. The pharynx consisted of an anterior cylindrical corpus that continued into a nearly indistinguishable isthmus and a round to ovoid-basal bulb with well-developed valves. In the female, the reproductive system was didelphic-amphidelphic, and the vagina provided with weak epiptygma (vulva forming folds with inner vaginal wall). The tail was cylindrical, slightly arcuate, and it had a spinneret with caudal glands in the tip (Figure 2C,D).

Aphelenchus, classified as fungal-feeder, presented lips slightly offset and the stylet lacking basal knobs. The esophageal glands overlapped the intestine dorsally and the tail of the female was short and bluntly rounded. The *Helicotylenchus* specimens had the characteristics of a semiendoparasite, with a vermiform spiral body when relaxed. The labial region was continuous to slightly offset, rounded or anteriorly flattened, and generally annulated. The stylets were of average size and the lateral field presented four lines. The median bulb of the esophagus had a rounded valve. In the females, one of the two genital branches was reduced and the vagina presented an epiptygma. The tail was curved dorsally, with a ventral process.

The omnivore nematode *Eudorylaimus* presented the lip region offset, and the

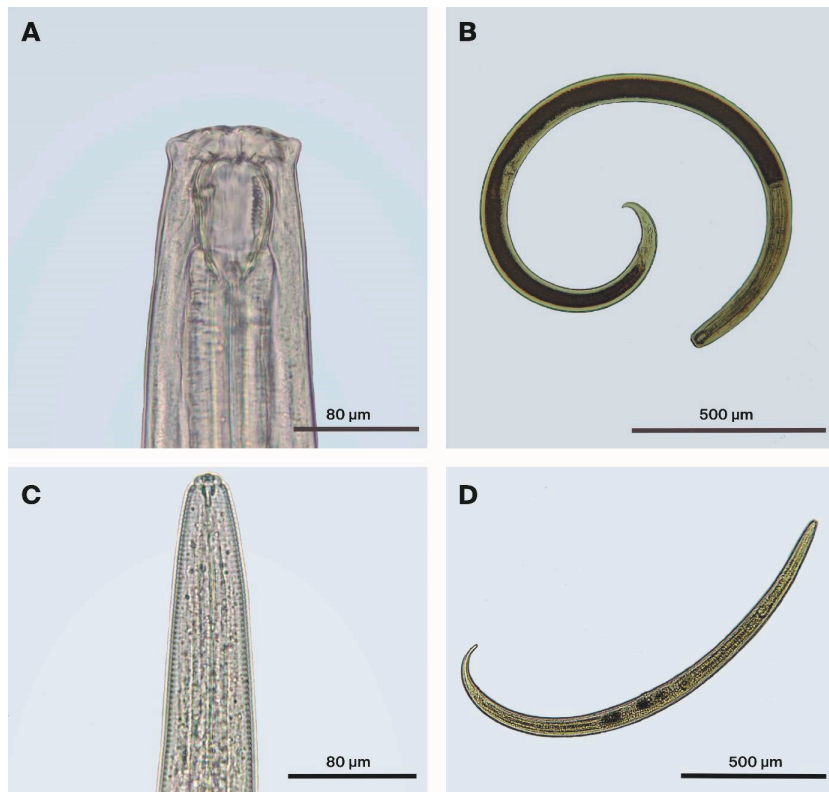


Figure 2. Nematodes collected from *Heterodermia diademata* in the Eco Área of Avellaneda, Buenos Aires province, Argentina. *Prionchulus* sp. female. A) Head. B) Entire body. *Plectus* sp. female. C) Head. D) Entire body.

Figura 2. Nematodos recolectados en *Heterodermia diademata* en la Eco Área de Avellaneda, provincia de Buenos Aires, Argentina. *Prionchulus* sp. femenino. A) Cabeza. B) Cuerpo completo. *Plectus* sp. femenino. C) Cabeza. D) Cuerpo completo.

odontostyle was about as long as the lip region width, with its aperture less than half its length and a single guiding ring. The anterior part of the pharynx was wide and gradually expanded. The genital system of the female was didelphic with reflexed ovaries, usually not reaching the junction of the oviduct and the uterus. The tail was short and conoid (Figure 3A,B).

Mesodorylaimus, an omnivore nematode, showed a truncated lip region offset by a slight depression. The odontostyle was 1.5 times the head width, with a simple rod-shaped odontophore and guiding ring. The pharyngeal expansion was gradual, widening at or after 50% of its length. The females presented a didelphic-amphidelphic genital system with reflexed, short ovaries not reaching or surpassing the oviduct-uterus junction and an elongated tail (Figure 3C,D).

Prionchulus presented a rounded lip region, offset by a weak depression, a funnel-shaped stoma with a typical predatory dorsal tooth of medium size located in the upper anterior

half of the buccal cavity and subventral denticles oriented anteriorly and forming a longitudinal row. The pharynx was muscular and cylindrical surrounding the basal part of the stoma. The genital system of the females was didelphic-amphidelphic, and the tail, conical, bent ventrally (Figure 2A,B).

Based on this diversity of feeding types, the ecological results found allowed us to classify the nematodes into 5 trophic groups: bacterivorous, fungivorous, plant-feeding, omnivores and predators (Table 1). Plant-feeder (semi-endoparasites) and fungivorous were the least abundant, each representing 1.2% of the total nematodes. The bacterivorous group was 16.3% of the total nematodes, predator 32.6% and omnivore were the most representative with 48.8% of the nematodes found. Nematodes were also classified according to colonizer-persistent values, finding representatives of classes 2-4 (Table 1). The values of the NINJA software gave a maturity index of MI=3.65. The structure and enrichment of the ecosystem shown by the

Table 1. Diversity of nematodes found in association with the substrate provided by *Heterodermia diademata* in the Eco Área of Avellaneda, Buenos Aires province, Argentina. Cp class is a value ranging from 1 to 5 and represents the strategy of free-living nematodes (cp=1 are colonizing nematodes, resistant to environmental disturbances; cp=5 are long-life nematodes, sensitive to environmental disturbances).

Tabla 1. Diversidad de nematodos encontrados en asociación con el sustrato proporcionado por *Heterodermia diademata* en la Eco Área de Avellaneda, provincia de Buenos Aires, Argentina. La clase cp es un valor que va de 1 a 5 y representa la estrategia de los nematodos de vida libre (cp=1 son nematodos colonizadores, resistentes a las perturbaciones ambientales; cp=5 son nematodos de larga vida, sensibles a las perturbaciones ambientales).

	C-p class	P-p class	Feeding type
<i>Acrobelus</i>	2	0	Bacterivorous
<i>Aphelenchus</i>	2	0	Fungivorous
<i>Eudorylaimus</i>	4	0	Omnivore
<i>Helicotylenchus</i>	3	3	Plant-feeder, semi-endoparasites
<i>Mesodorylaimus</i>	4	0	Omnivore
<i>Plectus</i>	2	0	Bacterivorous
<i>Prionchulus</i>	4	0	Predator

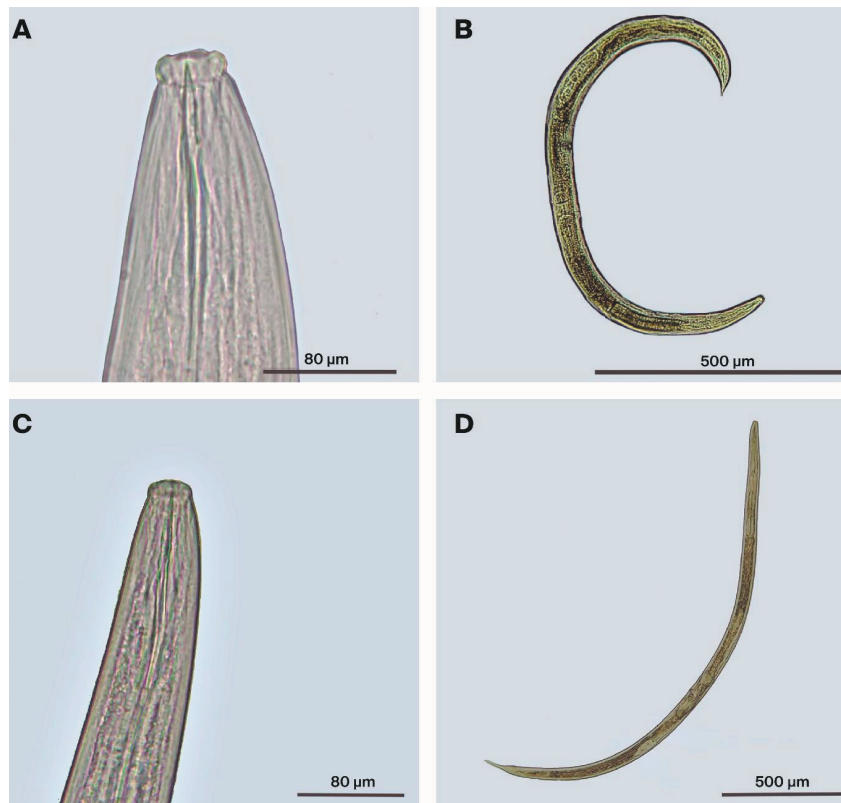


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substrate of *H. diademata* indicated values of EI=6.25 and SI=94.92, respectively.

DISCUSSION

In this case study, we found seven nematode genera inhabiting *H. diademata* of the Eco Área in Buenos Aires, Argentina, which is the first

record of this interaction for the country. These nematodes are usually found living on soil and mosses, and most studies describe their morphology but not the trophic role they occupy in the environment (Ryss et al. 2005; Cid del Prado 2012). Therefore, we set out to differentiate these groups of nematodes taxonomically and ecologically.

Lichens can support a wide range of organisms in their thallus (Gerson and Seaward 1977; Leinaas and Fjellberg 1985; Fröberg et al. 1993; Kumpula et al. 2004; Anderson 2014; Asplund et al. 2015), but little is known about the specific feeding strategies of nematodes in relation to lichens (Bokhorst et al. 2015). In a heteromorous thallus (such as in *H. diademata*), the medulla, formed by unpacked hyphae with spaces between, is used by organisms to move as if these were tunnels or as shelter (Huiskes et al. 1997; Asplund and Wardle 2017). There are complex food webs associated with the lichen thallus. For example, fungal-feeding nematodes likely feed on the lichen mycobiont, while bacterivorous nematodes – which can be abundant in lichen thalli (Bokhorst et al. 2015) – feed on various bacterial symbionts. Pennak (1978) proposed that the presence of nematodes in corticolous lichens may be due to their adaptability and high dispersibility by wind and animals.

Asplund and Wardle (2017) suggested that lichens may have a rich and specialized nematode fauna. Coinciding with this, we found 7 genera of nematodes associated with *H. diademata* representing all trophic levels of free-living nematodes: bacterivores (*Plectus* and *Acrobeles*), a fungivore (*Aphelenchus*), a plant-feeder (*Helicotylenchus*), omnivores (*Eudorylaimus* and *Mesodorylaimus*) and a predator (*Prionchulus*).

In this study, the trophic group of bacterivorous was almost exclusively represented by the genus *Plectus*, which is usually found in different types of soil, but has also been reported to inhabit mosses and lichens (De Geode 1993; Heidemann et al. 2014). Many species of this genus have a valvular apparatus, with transverse denticulate plates in the basal bulb, used to grind bacteria (Chaves et al. 2019). It has been observed that there is a relationship between traits of lichens and nematodes; in particular, Bokhorst et al. (2015) found that bacterivorous nematodes were common in lichens where the symbiosis was carried out with cyanobacteria, possibly associated with its high concentration of N. In this case, *H. diademata* has symbiosis with Chlorophyta, which would not have bacteria. However, lichens were also shown to harbor numerous communities of bacteria in their thallus (Bates et al. 2011). Therefore, the isolation and subsequent culture of the bacteria inhabiting the lichen can be used in feeding and preference assays to elucidate the diet of bacterivorous nematodes, such as

Plectus, in this environment. *Aphelenchoides* – a fungivorous nematode – was cited in lichen samples (Gadea 1976), but in this study it is unknown whether it feeds on the fungal part of the lichen or if its diet is based on a portion of the associated microbiota.

Mesodorylaimus feed on a wide range of organisms, such as invertebrates, algae, fungi and bacteria (McSorley 2012). For instance, *Mesodorylaimus bastiani* has shown predatory behavior in other nematodes, ingesting the body content through the odontostyle (Bilgrami 2008). According to Spaul (1973), direct observations suggest that a species of *Mesodorylaimus* may feed upon coccoid algae and dead collembola. *Eudorylaimus carteri* was cultured by Nielsen (1949) in water with different substrates such as algae, bacteria and moss leaves, while it was hypothesized that *Eudorylaimus antarcticus* feed on algae (McSorley 2012). *Eudorylaimus noterophilis* is a predator in colonies of *Panagrelus redivivus* (Jairajpuri and Ahmad 1982). The *Eudorylaimus* and *Mesodorylaimus* found may be feeding from algae that form the lichen, or preying on other nematodes. Future studies would help to understand the diet of this trophic group of nematodes sensitive to environmental disturbances.

There is also a relatively high abundance of predator nematodes, at least in epiphytic foliose lichens (Bokhorst et al. 2015), and these are likely to feed on various lichen-associated microfauna. *Prionchulus* is a genus that feeds on a variety of organisms and other nematodes due to its conspicuously large-mouth cavity with one dorsal tooth (Vera-Morales et al. 2022). Predatory nematodes could regulate the populations of other nematodes when these begin to grow exponentially due to the presence of excess nutrients (Roy and Borah 2020). Further analyses are needed to determine the diet of this trophic group and to evaluate its capacity as a regulatory predator, representative of the top of the nematode food web. Due to their low abundance (n=1), the bacterivorous nematode *Acrobeles* and the phytophagous *Helicotylenchus* were not representative for discussing their results.

The NINJA system was developed to measure soil quality and soil health using nematodes as bioindicators (Sieriebriennikov et al. 2014). In this work, we tested this system in different media/substrates, considering *H. diademata* as an environment for nematodes. This software calculates all indices based on the abundance of taxa in the samples, indicating ecosystems

that tend toward equilibrium or those that present environmental disturbances. The maturity index and structure index values are novel, reflecting the capacity of *H. diademata* as a micro-habitat refuge of nematode diversity. MI values of MI are between 1 and 3-4, with the highest values being representative of undisturbed environments (Salas and Achinelly 2020). The MI value found was 3.65, indicating an undisturbed shelter for the nematode microhabitat at this site within the eco-region. Our results show a low enrichment index and a high structure index (EI=6.25 and SI=94.92). These values indicate a trophic interaction in which primary consumers serve as food for the development of omnivorous and predatory nematodes (Talavera et al. 2021). The fact that no nematodes of the cp1 classes (or r-strategists, as they can be considered) were found may indicate that these nematode communities are not of an early stage in succession (Salas 2019). Thus, the structure of the trophic community of nematodes may indicate a balance between primary consumer nematodes (opportunistic

nematodes of nutrient enrichment) and omnivorous and predatory nematodes (i.e., nematodes sensitive to disturbances, pest suppressors and population growths of primary consumers). These results are encouraging for future research on the comparison of diversity between other species of lichens and their environmental seasonality. This could be used in ecological studies to establish relationships between the capacity of lichens to host micro-fauna and their condition as environmental indicators.

Studies on this association are scarce in the world and there are no reports on this kind of interaction in Argentina. In this first approach we were able to find nematodes with various feeding habits and we expect to improve the knowledge of the association between lichens and nematodes in future studies.

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