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Original Paper

Secrets beneath the soil: recovery of fern spores as a strategy of biodiversity conservation in Punta Lara Nature Reserve (PLNR), Argentina

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Abstract

The recovery of soil spores is a strategy to strengthen in decline or disappeared populations from natural environments. In this work, we analyzed 25 soil samples extracted from a patch of gallery forest in an "albardón" of Punta Lara Reserve, Buenos Aires. The samples were distributed in 50 Petri dishes, 25 exposed to controlled temperature and light and another 25 kept in darkness. To contribute to the identification of gametophytes and sporophytes, spores of the local species were cultured *in vitro*. In 18 months of trial, the appearance of gametophytes and sporophytes was observed, in a greater proportion those belonging to a dominant species in the community: *Doryopteris concolor*. Numerous gametophytes and sporophytes from *Gastoniella chaerophylla* were also obtained, a taxon not found in the "albardón" for two years. The germination index (GI) was estimated and the morphological characteristics of the gametophytes and sporophytes were recorded. This is the first contribution to the knowledge of the spore banks in Argentina, in a protected area where several threats put at risk the survival of native species. The bases to implement methods of *ex situ* and *in situ* conservation of native ferns are provided.

Key words: conservation, ferns, gametophytes, spore bank, soil.

Resumen

La recuperación de esporas del suelo es una estrategia para reforzar poblaciones en disminución o desaparecidas de los ambientes naturales. En este trabajo se analizaron 25 muestras de tierra extraídas de un parche de selva en galería en un albardón de la Reserva Punta Lara, Buenos Aires. Las muestras fueron distribuidas en 50 cápsulas de Petri, 25 expuestas a temperatura y luz controladas y otras 25 mantenidas en oscuridad. Para contribuir a la identificación de los gametofitos y esporofitos se cultivaron *in vitro* esporas de las especies del lugar. En 18 meses de ensayo se observó la aparición de gametofitos y esporofitos, en mayor proporción los pertenecientes a una especie dominante en la comunidad: *Doryopteris concolor*. Se obtuvieron además numerosos gametofitos y esporofitos de *Gastoniella chaerophylla*, un taxón no encontrado en el albardón desde hace dos años. Se estimó el índice de germinación (IG) y se registraron las características morfológicas de los gametofitos y esporofitos. Esta es la primera contribución al conocimiento de los bancos de esporas en Argentina, en un área protegida donde diversas amenazas ponen en riesgo la supervivencia de especies nativas. Se brindan las bases para implementar métodos de conservación *ex situ* e *in situ* de los helechos nativos.

Palabras clave: conservación, helechos, gametofitos, banco de esporas, suelo.

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Introduction

Natural soil germplasm banks are a useful tool to delve into the floristic diversity of a plant community and to implement, if necessary, population reinforcements while maintaining genetic variability (*i.e.*, Ibars & Estrelles 2012). In ferns particularly, soil spore banks (as occurs with seed banks of the spermatophytes) contribute to ensure the regeneration of populations and recolonization of habitats after disturbances (Chesson 1994; Dyer 1994; Thompson 2002; Auld & Denham 2006; Hock *et al*. 2006; Paul *et al*. 2014).

By analogy with the concept of persistent seed banks in the soil, spore banks are defined by the existence of spores that remain viable in the soil or substrate for long periods of time (generally for more than a year) (Nondorf *et al*. 2003; Hock *et al*. 2006; Paul *et al*. 2014). The soil banks allow the survival of plant species, especially in conditions environmentally adverse where natural selection favors those which can delay germination until the environmental conditions are adequate (Fenner & Thompson 2005; Hock *et al*. 2006; Paul *et al*. 2014).

A manner to conserve biological diversity, understood as that of ecosystems, species and genepool, is by creating protected areas. The Punta Lara Natural Reserve (PLNR) is located on the side of the La Plata river, Buenos Aires Province, and constitutes the core area of the Pereyra Iraola Biosphere Reserve UNESCO (Barbetti 2008). From a phytogeographic point of view, the Reserve is included in the Chaco Domain, Oriental Pampean District of the Pampean Province (Cabrera 1976; Arana *et al*. 2017). In this zone, the proximity to the river determines a great variety of ecological niches. According to Cabrera (1971), the main plant communities developed in this area are the gallery forests, the Espinal forests, flooded scrublands and the grasslands. The particular floristic association of the forests that borders the internal streams in the Reserve comprises arboreal species such as *Blepharocalyx salicifolius* (Kunth) O.Berg, *Allophylus edulis* (A.St.-Hil., A.Juss. & Cambess.) Hieron. *ex* Niederl., *Ocotea acutifolia* (Nees) Mez., *Pouteria salicifolia* (Spreng.) Radlk. and *Lonchocarpus nitidus* (Vogel) Benth. They grow accompanied by climbing species, epiphytes, herbs and shrubs that can be also found in the forests of southern Brazil (Closs de Marchi & Jarenkow 2008; Guerrero *et al*. 2018) and northeastern Argentina. This floristic composition is also very similar to that of the marginal forests of the Uruguay River and its tributaries (Parodi 1943; Burkart 1963; Grela 2004).

Regarding fern diversity, about 21 native taxa inhabit in the Reserve, among which the most frequent are: *Goniopteris burkartii* C. Chr. *ex* Abbiatti, *Asplenium ulbrichtii* Rosenst., *Microgramma mortoniana* de la Sota and *Blechnum auriculatum* Cav. (=*Blechnum australe* L. subsp. *auriculatum* (Cav.) de la Sota) (Cabrera 1939; Cabrera & Dawson 1944; Moschione 1987; Giudice *et al*. 2011; Ponce *et al*. 2016). In this zone, the specific diversity of ferns shows a marked influence of the flora from southern Brazil, with the rivers Paraná and Uruguay acting probably as migratory routes along the gallery forests (de la Sota 1973; Giudice *et al*. 2011).

The communities of the Punta Lara Reserve are exposed to the adverse effects of the anthropic impact (about 1 million inhabitants in the surrounding area), which entails among other effects habitat reduction, placing at risk the survival of many native plant species (Delucchi 2006). The native flora is affected also by the introduction of exotic species such as the privet (*Ligustrum lucidum* W.T.Aiton) and the blackberry bush (*Rubus ulmifolius* Schott), which have more aggressive competitive strategies (Giudice *et al*. 2011). Both threats represent a problem when defining conservation strategies in this area. In this sense, soil spore banks have a major role in the conservation of fern taxa in danger of extinction (Dyer & Lindsay 1992, 1996; Dyer 1994).

Since 2006 this research group has been developing a project to explore the diversity and reproductive biology of native ferns in Punta Lara Reserve, based on studies on spore germination and sporophyte development, as a contribution to their conservation (*i.e.*, Giudice *et al*. 2011, 2014; Ramos Giacosa *et al*. 2014, 2017; Luna *et al*. 2016; Gorrer *et al*. 2018). Until the start of this study, there were no other investigations that addressed the soil spore banks in this country. Therefore, our main objective is to advance in the evaluation of the role of the soil as a reservoir of fern biodiversity, as well as to explore new strategies for the conservation of the flora in a protected area. A fundamental part would be in this case to promote the benefits of these trials for better preservation of biodiversity in areas with high human impact.

Materials and Methods

Study site

Sampling was carried out in a site of the "albardón" called "La Araucaria" located at the Punta Lara Natural Reserve (34º47'S, 58º01'W) (Fig. 1a). The weather data for this zone (expressed in mean annual values) are: precipitation 994 mm, temperature 16.5 °C and humidity 80%. The warmest month of the year is January with an average maximum temperature of 30.5 °C whereas July is the coldest, with an average minimum of 7.3 °C.

An "albardón" is a low elevation formed in the past by deposition of sandy material because of river fluctuations. In "La Araucaria", the tree species typical of the gallery forest develop along with others characteristic of xerophylous forests, such as *Jodina rhombifolia* (Hook. & Arn.) Reissek. Although the gallery forest is a system highly dependent on the dynamics of nutrients resulting from the flood pulses of the Río de la Plata basin (Frangi 1993; Bó & Malvárez 1999), this "albardón" does not undergo hydromorphism processes. Thus, nowadays there is rather a pedological stability given by processes of melanization and argiluviation, due to the low fluctuations and income of the water from the river.

Structurally, the patch of forest consists of an arboreal stratum conformed by native species of the gallery forest (Fig. 1b), and some exotic ones such as *Ligustrum lucidum* (Dascanio & Ricci 1988). Under this canopy develop various populations of ferns. Two of them are dominant: *Asplenium ulbrichtii*, whose populations consist of grouped individuals (Fig. 1c-d) and *Doryopteris concolor* (Langsd. & Fisch.) Kuhn, whose individuals are scattered distributed (Fig. 1e-f). A population of *Adiantopsis chlorophylla* (Sw.) Fée is represented by only two individuals that have not developed sporangia since 2015 (Berrueta, personal observation). The epiphytic *Microgramma mortoniana* grows on *Pouteria salicifolia* trees branches and also on ground fallen branches. Populations of *Blechnum auriculatum* and *Equisetum giganteum* L. are also found, both with individuals scattered distributed. Occasionally, two isolated sporophytes of *Gastoniella chaerophylla* (Desv.) Li Bing Zhang & Liang Zhang were registered during the years 2007 and 2009 (Luna, personal observation). As in several zones of the Reserve, the invasive species *Rubus ulmifolius* is present in the "albardón".

Soil Sampling

Twenty-five samples (250 g each) were extracted randomly with a shovel in the "albardón", in a plot 25 × 25 meters digging to a depth of 10 cm from the surface. Samples were taken during the winter (August 2016) just before the main spore release season. They were placed in polyethylene bags with hermetic closure and transported to the laboratory. The 25 soil samples were fractionated and distributed in 50 previously sterilized Petri dishes 9 cm in diameter (soil layer thickness ca. 1 cm in each capsule). Half of them were watered, wrapped in film and placed in a cultivation chamber under controlled conditions of temperature (21–24 °C) and light (white fluorescent illumination 28 μmol m^{-2} s^{-1}, a lamp for each shelf, with a photoperiod of 12 hours). The other 25 dishes were watered, wrapped in foil and kept in darkness.

Additionally, a sample of 1 kg of soil was collected randomly in the plot to determine the pH, conductivity and texture. This analysis was realized at the Institute of Geomorphology and Soils, Center for Soil and Water Research for Agricultural use (IGS-CISAUA). The analysis of organic matter was performed using the Walkley & Black (1934) method, wet oxidation with $H_2Cr_2O_7$ and titration of excess with $FeSO_4$. Textures were determined by Bouyoucos (densimetric) pH in saturated paste in a 1: 1 ratio and the conductivity analyses was performed on the saturated paste extract.

Estimation of gametophyte coverage

In order to estimate the percentage of spore germination in soil cultures, a germination index (GI) modified by Ramírez *et al*. (2000) was used. This index is obtained indirectly according to the degree of gametophyte's coverage, using a subjective percentage estimate, so that the number of individual gametophytes is not taken into account. The scale is an adaptation of the abundance-coverage of Braun-Blanquet and takes into account the coverage of the gametophytes developed in a grid, measured as a percentage (Tab. 1). A grid of 1 cm^2 was used, in which 5 quadrants were chosen for each replica. The capsules were introduced in an incubator and monthly controls were carried out until GI saturation. The observations were made under a stereoscopic microscope (Nikon SMZ 1000).

A non-parametric ANOVA (Kruskall-Wallis test) was performed using the software Statistica 7.1 to observe possible groupings in the controls.

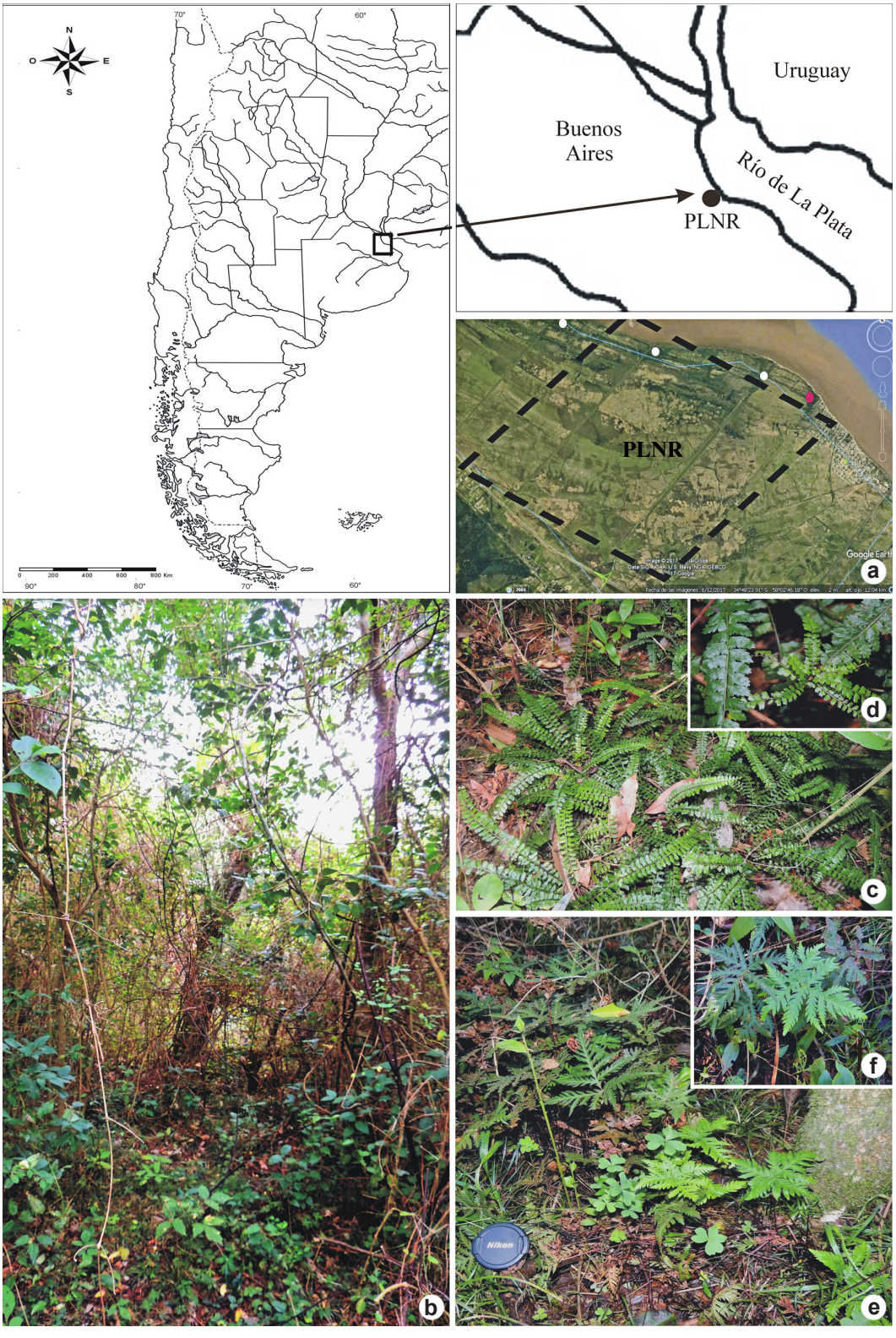

Figure 1 – a-b. Study area – a. localization of Punta Lara Natural Reserve (PLNR) in Argentina, at the margins of the La Plata River; b. study area in the albardón "La Araucaria". c-f. populations of the most common ferns in the study area – c-d. *Asplenium ulbrichtii*; e-f. *Doryopteris concolor*.

Table 1 – Germination index (GI) scale.

Germination Index (GI)	Estimate percentage of gametophyte coverage
0	no gametophytes
1	< 5 %
2	5–25%
3	25–50%
4	50–75%
5	> 75%

A reversion of the GI values in 16 controls (when GI saturated) was also performed.

Taxonomic identification of gametophytes

To facilitate the taxonomic identification of the gametophytes observed in the soil samples, spores of species present in the study area were collected and cultivated *in vitro* (except for *A. chlorophylla*, as no spores were obtained during the study). Previously achieved information on the gametophytic development of some species was also consulted for gametophyte identification (Ramos Giacosa *et al*. 2014, 2017; Luna *et al*. 2016; Gorrer *et al*. 2018).

Spores were sterilized in an aqueous solution 10% of NaClO (5 gl/l) for three minutes, rinsed three times with distilled water and germinated in Petri dishes containing Dyer medium (Dyer 1979) and Difco Bacto-agar (7 g/l). Dishes were kept under laboratory conditions of temperature (22–24 °C) and light (white fluorescent illumination 28 µmol m^{-2} s^{-1}) with a photoperiod of 12 hours.

Results

Soil analysis

The samples have an acid pH (5.3) and low conductivity (0.02 d/sm) with a sandy-loam structure (72.81% sand, 19.87% silt, 7.32% clay).

Gametophyte coverage

The Kruskall-Wallis test for nonparametric data indicates that the inspections or controls do not present significant differences between them until control 10 (month 10); controls 11 and higher are significantly superior to the rest ($H = 322,96$; pvalue $\leq 0,0001$). In the analysis of trends, a logarithmic trend of the germination index (GI) was observed, showing its saturation after control 10 (Fig. 2). At the control number 13, 95% of the capsules reached a GI of 5 when the gametophytes covered the entire surface of the 25 capsules exposed to light. At the control 16, the GI saturated in all samples.

No gametophytes developed in samples kept in the dark.

Taxonomic identification of gametophytes developed in soil samples

Gametophytes assignable to *Doryopteris concolor* developed in all soil samples (35 in total). By comparison with those obtained *in vitro*, they acquired a typical cordate shape and showed no trichomes (Fig. 3a-d). Their rhizoids were hyaline (Fig. 3b). Buds formed on the margins of the gametophyte after the emergence of the antheridia (Fig. 3a). Later, all gametophytes became bisexual (Fig. 3c-d).

In 10 soil samples, helical gametophytes characteristic of *Gastoniella chaerophylla* were found (Fig. 3e-f). According to Luna *et al*. (2016),

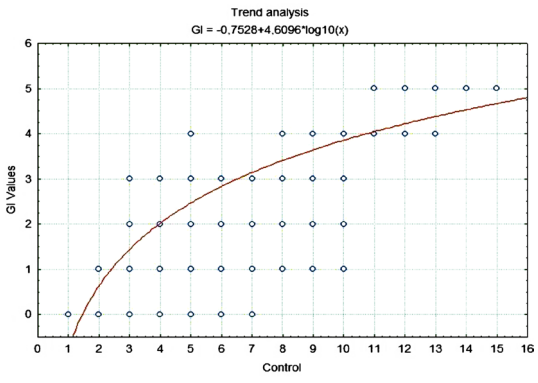

Figure 2 – Trend analysis of germination index (GI) in 16 monthly controls.

a uniseriate filament 3–4 cells in length develops first, followed by apex division into two directions. As development progresses, the prothallus acquires an asymmetrical spatula shape, bending later around the growth zone acquiring thus a funnel shape. A total of 16 gametophytes with this peculiar prothallus development were registered (Fig. 3f).

Occasionally, gametophytes with characteristics attributable to *Asplenium ulbrichtii* were found in one soil sample (only two gametophytes) (Fig. 4a-d). Under *in vitro* conditions, *A. ulbrichtii* develops first a filament 5–6 cells in length (Fig. 4b). Then, successive divisions of the apical cell begin to produce a

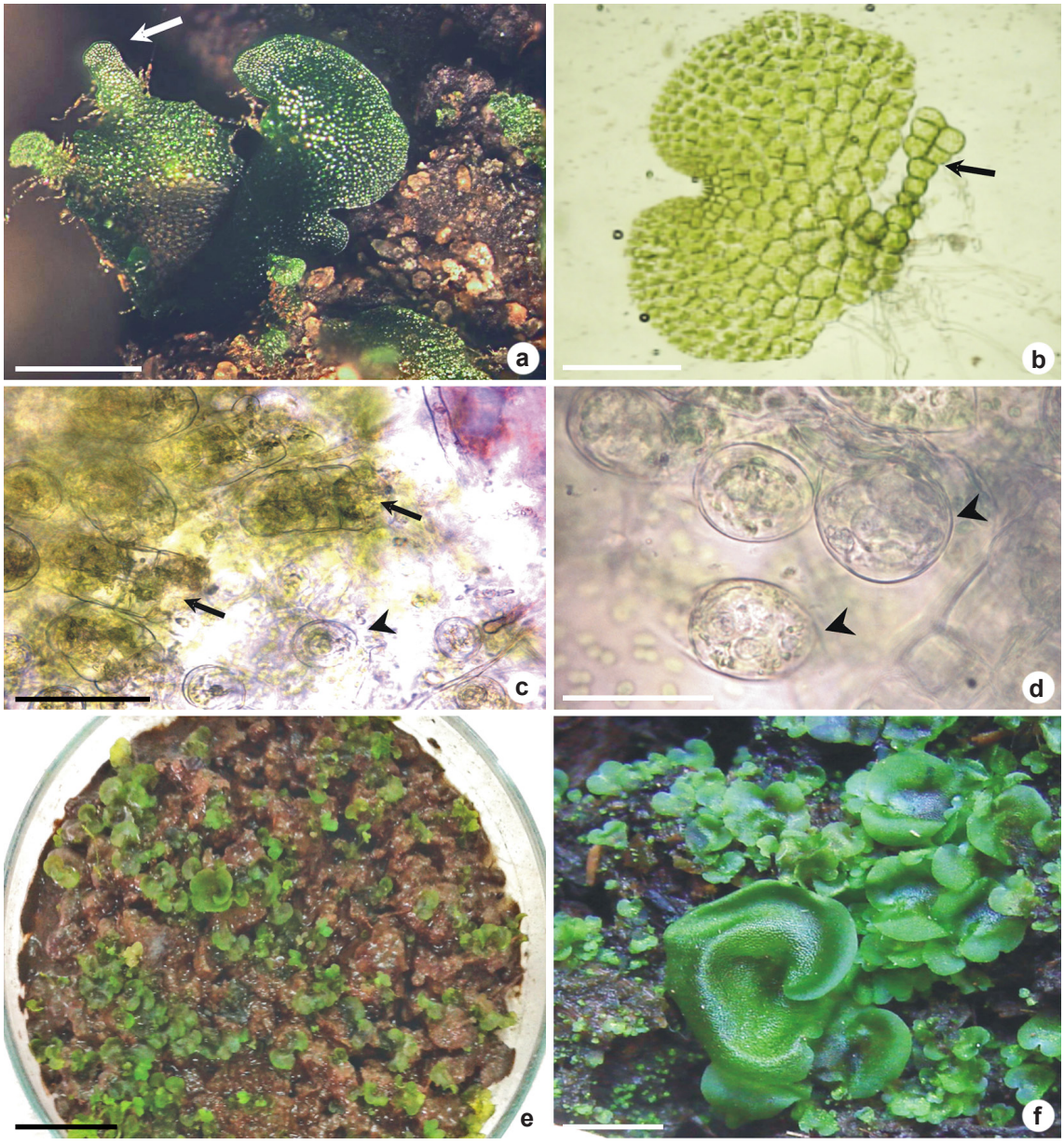

Figure 3 – a-f. Development of gametophytes – a-d. *Doryopteris concolor* – a. gametophytes with buds in soil sample (arrow); b. cordate gametophyte with buds (arrow) grown *in vitro*; c. bisexual gametophyte with archegonia (arrows) and antheridia (arrowhead); d. detail of antheridia (arrowheads); e-f. *Gastoniella chaerophylla* – e. gametophytes in soil sample. f. detail of funnel-shaped prothallus. Bars: a = 0.5 mm; b, c, d = 50 µm; e = 2 cm; f = 1 cm.

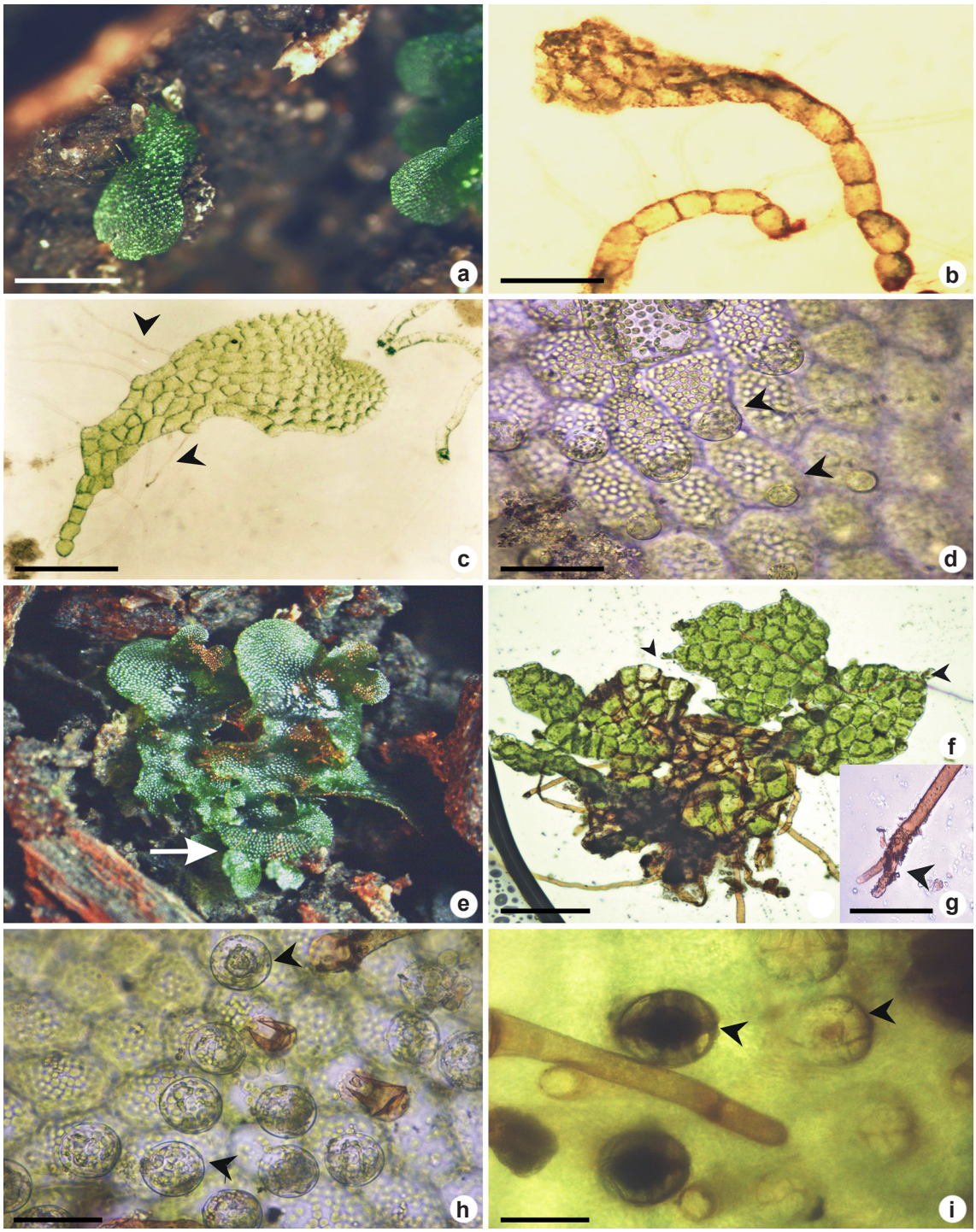

Figure 4 – a-i. Development of gametophytes – a-d. *Asplenium ulbrichtii* – a. spatulate gametophyte in soil sample; b. earlier stage of spatulate gametophyte grown *in vitro*; c. a more advanced stage with hyaline rhizoids (arrowheads); d. gametophyte with antheridia (arrowheads); e-i. *Microgramma mortoniana* – e. gametophytes with buds (arrow) grown in soil sample; f. gametophytes with unicellular hairs on the margins (arrowheads) and brown rhizoids; g. detail of bifurcated rhizoid (arrowhead); h. antheridia (arrowheads); i. archegonia (arrowheads). Bars: a = 0.5 mm; b, c, d, g, h, i = 50 µm; e = 5 mm; f = 100 µm.

spatulate gametophyte with hyaline rhizoids (Fig. 4c). Gametophytes obtained in both, in soil and *in vitro* cultures, produced only male reproductive structures (antheridia) (Fig. 4d).

Also, only two gametophytes such as those described by Gorrer *et al*. (2018) for *Microgramma mortoniana* were observed in soil cultures (Fig. 4e). They were cordiform and showed unicellular trichomes and brown rhizoids, the latter with bifurcated terminal ends in some instances (Fig. 4f-g). The gametophytes produced buds (Fig. 4e) that were detached after the formation of antheridia (Fig. 4h). Afterwards, they developed archegonia (Fig. 4i).

They were no registered gametophytes of *A. chlorophylla*, *B. auriculatum* and *E. giganteum*, common species in the area.

Sporophytes developed in soil cultures

After 18 months, sporophytes of *D. concolor* and *G. chaerophylla* were visualized (Fig. 5a-e). Those of *D. concolor* were the most abundant (25 in total), and they were identified by the appearance of a first frond with orbicular leaf blade (Fig. 5a-b) and a brown petiole (Fig. 5c). In the case of *G. chaerophylla* only five sporophytes developed, these characterized by their fronds with palmate leaf blades (Fig 5d-e). Only one sporophyte of *M. mortoniana* was registered while samples were inspected, this one consisting in an oblong short-petiolate frond (Fig. 5f). No sporophytes of *A. ulbrichtii* developed during the conduct of this study (only male gametophytes were registered).

Discussion

The gametophytes developed in soil cultures belonged to the fern species inhabiting the "albardón". This could indicate preliminarily that the dispersion of other species from different zones of the Reserve via spores seem to be infrequent. The knowledge about spore dispersion at long distances is of relevance because this phenomenon makes possible the colonization of new sites and also gene flow between different populations, thus preventing the reproduction of gametophytes from the same mother plant (Dyer & Lindsay 1992; Simabukuro *et al*. 1998, 1999, 2000; Esteves & Dyer 2003; Groot *et al*. 2011, 2012; Coelho *et al*. 2017).

During soil cultures, gametophytes of *D. concolor* were the most abundant, as well the number of sporophytes developed from them, which indicates that a relatively high number of spores of this species remains viable in the soil for at least one year.

On other hand, the few observed gametophytes of *A. ulbrichtti* developed antheridia but not archegonia. In the natural habitats, we observed that the new sporophytes originated mostly asexually from foliar buds. The distribution of the individuals forming grouped populations seems be an indicator of this phenomenon. Some authors, such as Page (2002), consider that buds production is an indicator of stable environmental factors, as those registered in the "albardón", where no permanent river floods occur. Currently, deeper studies on the reproductive biology of the ferns that grow in this site are being developed, however this information exceeds the scope of this work.

It should be noted that during soil cultures many gametophytes of *G. chaerophylla* arose, although no sporophytes were recorded in "La Araucaria" site since 2014 (Luna, personal observation). This species is characterized by its annual sporophytes and the production of tubercles from the gametophytes, which persist through dry periods (Luna *et al*. 2016.). The development of gametophytes during soil cultures indicates that the spores of *G. chaerophylla* remain viable for at least 2 years (taking into account the sampling date), and that under controlled conditions of light and temperature they are able to germinate.

Concerning *M. mortoniana*, the scarce emergence of gametophytes in soil cultures would be an indicator of its prevalent mode of propagation through rhizomes (Berrueta, personal observation). Also, as an epiphytic species, it is assumed that the spores fall mainly on the branches of the supporting plants. During gametophyte's identification, new characters for this species such as bifurcated rhizoids were observed, in addition to those reported by Gorrer *et al*. (2018).

As mentioned previously, the few individuals of *A. chlorophylla* that grow in this "albardón" site have not been fertile for the last 3 years. Gametophytes and sporophytes attributable to this species were not observed during soil cultures, which allow us to assume that, if spores existed in the soil, they lost their viability. The reproduction of the ferns can be conditioned also by the presence of the invasive exotic *Rubus ulmifolius*, which grows profusely in the sampling zone, thus limiting the amount of light reaching the herbaceous stratum. Perhaps due to this, many spores do not germinate in the natural environments as they do when they are cultured under laboratory conditions. As in the photoblastic seeds, it is known that the germination of fern spores is controlled in various species by a phytochrome, which detects light changes in the

environment (Raghavan 1989; Banks 1999; Furuya *et al*. 1997; Kodama *et al*. 2008; Tsuboi *et al*. 2012). The absence of germination in all the trials kept in darkness suggests this phenomenon. It has been shown by various authors that some spores germinate in forest clearings, a condition that favors gametophyte and sporophyte grow (Smith 1995, 2000; Pérez-García *et al*. 2007).

Our findings in *D. concolor* and *G. chaerophylla* are in agreement with Ramírez-Trejo *et al*. (2004) in that the persistence of soil spore banks determines the natural capacity of wild populations for *in situ* regeneration. Dyer (1994) raised the idea that a mode to increase population's survival and the genetic diversity (and perhaps even to recover lost populations), is by reintroducing plants derived from spore banks or by stimulating regeneration from *in situ* spore banks. Estrelles *et al*. (2001) also argue that soil spore banks can be very useful to strengthen threatened species with very small populations. Currently the sporophytes of *D. concolor* and *G. chaerophylla*, obtained from the soil spore bank, are being rusticated to be reinserted into the natural environments.

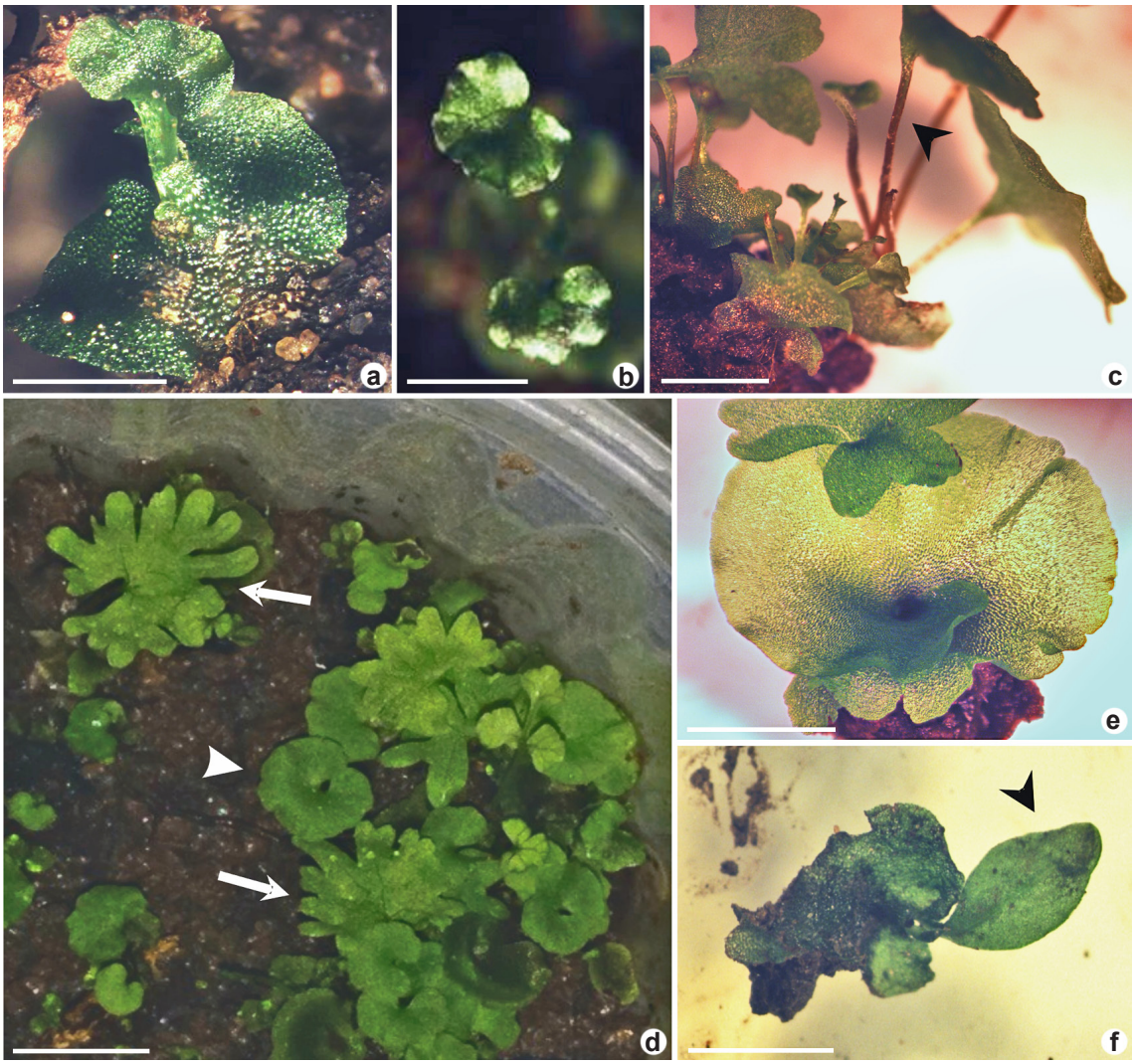

Figure 5 – a-f. Sporophytes developed in soil samples – a-c. *Doryopteris concolor* – a. sporophyte showing the first frond with orbicular leaf blade; b. detail of leaf blades; c. brown petioles (arrowhead); d-e. *Gastoniella chaerophylla* – d. first fronds with palmate leaf blades (arrows). In the same capsule grows *Doryopteris concolor* (arrowhead); e. detail of funnel-shaped gametophyte and sporophyte with palmate leaf blade; f. *Microgramma mortoniana* – first frond spatulate, short-petiolated (arrowhead). Bars: a, b, c = 5 mm; d, e, f = 1 cm.

Regarding soil characteristics, samples from the "albardón" showed an acid pH, more than those from others sites where ferns grow (Berrueta *et al*. unpublished data), along with a lower conductivity (lower water content) and a greater amount of organic matter. This soil type seems to be optimal for the development of *A. ulbrichtii* populations forming profuse colonies, and also for the establishment of species such as *A. chlorophylla* and *D. concolor*, which until now were only registered in the "albardón". From our observations, a need arises to deepen on the associations between the soil types and the fern populations that develop on them, as well as on the knowledge of the microorganisms that inhabit them.

This is the first contribution on spore banks in Argentina in an area with diverse threats that put at risk the survival of many native species. We intend to provide tools for fern conservation in our country and to encourage ecological studies in ferns.

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