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Gametophyte development and conservation of *Ctenitis submarginalis* (Dryopteridaceae) in Buenos Aires Province, Argentina

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Summary: The spore germination and gametophyte development of *Ctenitis submarginalis* from Punta Lara Natural Reserve, Buenos Aires province were studied as a contribution to the conservation of the species in this protected area. Spores collected from natural populations were sowing in Dyer medium and germinated in six day with a percentaje of 88 % fifteen days after sowing. The germination is *Vittaria* type and the gametophyte development is *Aspidium* type. The gametophyte of *Ctenitis submarginalis* is cordate, with a deep notch and well developed and symmetric wings. The gametangia appear 50 days after sowing. The sporophyte emerges 190 days after sowing and two different hairs were observed in the first lamina. The in-vitro spore germination is a successful method for obtaining numerous sporophytes of *Ctenitis submarginalis*. The sporophytes obtained were gradually acclimated, transplanted to its natural habitat in the reserve and will be monitored frequently to contribute to the conservation of the species.

Key words: Conservation, *Ctenitis*, Dryopteridaceae, fern, gametophyte.

Resumen: Desarrollo del gametofito y conservación de *Ctenitis submarginalis* (Dryopteridaceae) en la provincia de Buenos Aires, Argentina. Se estudió la germinación de las esporas y el desarrollo del gametofito de *Ctenitis submarginalis* de la Reserva Natural Punta Lara, provincia de Buenos Aires como contribución a la conservación de la especie en ésta área protegida. Las esporas colectadas de las poblaciones naturales fueron sembradas en medio de Dyer y la germinación se produjo a los seis días, con un porcentaje de germinación del 88 % a los 15 días después de la siembra. La germinación es del tipo *Vittaria* y el desarrollo del gametofito tipo *Aspidium*. El gametofito de *Ctenitis submarginalis* es cordado, con una profunda escotadura y alas simétricas bien desarrolladas. Los gametangios aparecen a los 50 días después de la siembra. El esporofito emerge a los 190 días después de la siembra y dos tipos diferentes de pelos se observaron en la primera lámina. La germinación in-vitro a través de esporas es un método exitoso para la obtención de numerosos esporofitos de *Ctenitis submarginalis*. Los esporofitos obtenidos fueron gradualmente aclimatados, transplantados a su hábitat natural en la reserva y serán periódicamente monitoreados para contribuir a la conservación de la especie.

Palabras clave: Conservación, *Ctenitis*, Dryopteridaceae, helechos, gametofitos.

Introduction

The genus *Ctenitis* (C. Chr.) C. Chr. (Dryopteridaceae) comprises about 125 species distributed in tropical, subtropical and temperate areas of the Old and New world (PPGI, 2016). About 75 species grows in America from the U.S.A. south to Argentina. In the last country only two species were found: *Ctenitis ampla* (Humb. & Bonpl. ex Willd.) Ching and *C. submarginalis* (Langsd. & Fisch.) Ching (de la Sota, 1977; Tryon & Tryon, 1982; Mickel & Smith 2004; Zuloaga *et al.*, 2008). This genus differs to other genera of Dryopteridaceae by the costae raised on the upper surface of the segments and the presence of distinctive short multicellular trichomes, especially on the adaxial rachises, costae and minor axes (Tryon & Tryon, 1982; Mickel & Smith, 2004).

Ctenitis submarginalis (Langsd. & Fisch.) Ching grows from the south-east of the U.S.A. to Argentina,

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where the species reaches its limit of austral distribution in the riverside of La Plata River, Buenos Aires province. In this area the Punta Lara Natural Reserve, a relict of the southernmost riparian forest in the world, is present.

This protected area is situated from 12 km north to La Plata City (34º 47'S - 58º 01' W) and the flora is threatened by the anthropic expansion, the introduction of exotic species and the extraction of ferns with ornamental purposes that decrease the natural populations (Giudice *et al.*, 2011). *Ctenitis submarginalis* is growing in a small area of this protected reserve and isolated individuals can be found.

A good conservation strategy of the threatened flora of an area constitutes the in-vitro propagation to obtain sporophytes in the laboratory, to the reintroduction or reinforcement (IUCN, 1998) the populations of an area. This method of plant conservation was carried out only in a few ferns species of other countries, as *Marsilea* L. in Spain (Estrelles *et al.*, 2001), *Tectaria* Cav. in Australia (Ashmore *et al.*, 2011), *Woodsia* R. Br. in Britain and Estonia (McHaffie, 2006; Aguraiuja, 2011) and for the first time in Argentina in the genus *Thelypteris* Schmidel *(sensu lato)* in Punta Lara Natural Reserve (Ramos Giacosa *et al.*, 2012).

In-vitro spore germination is a reliable method for the propagation of ferns, and this procedure is being carried out widely, often in conjunction with cryopreservation, for the conservation of many species (Baker *et al.*, 2014). The knowledge of the ferns reproductive biology and appropriate methods for propagation, planting out and survival of the individuals are essential for a conservation program.

Another important aspect is that the study of the spore germination and gametophytic phase could be an important character to be included in systematic or phylogenetic studies.

The gametophytic development of only a few species of *Ctenitis* it is known. Kaur and Devi (1976) studied the gametophytes of four *Ctenitis* species. The species analyzed by these authors have a *Vittaria* type germination and *Aspidium* type prothallial development. Later, Meng *et al.* (2008) analyzed gametophytic development of two species of *Ctenitis* from China.

The aim of this work is to study the spore germination, gametophyte development and young sporophytes of *Ctenitis submarginalis* to give characters for systematic or phylogenetic purposes and to test if the in-vitro spore germination could be an effective method to propagate this fern for conservation programs in Buenos Aires province, Argentina.

Material and Methods

Fertile fronds were collected from natural populations growing in Punta Lara Natural Reserve, provincia de Buenos Aires, Argentina (34º 47' S y 58º 01' O) in March 2013 (Fig. 1, A, B).

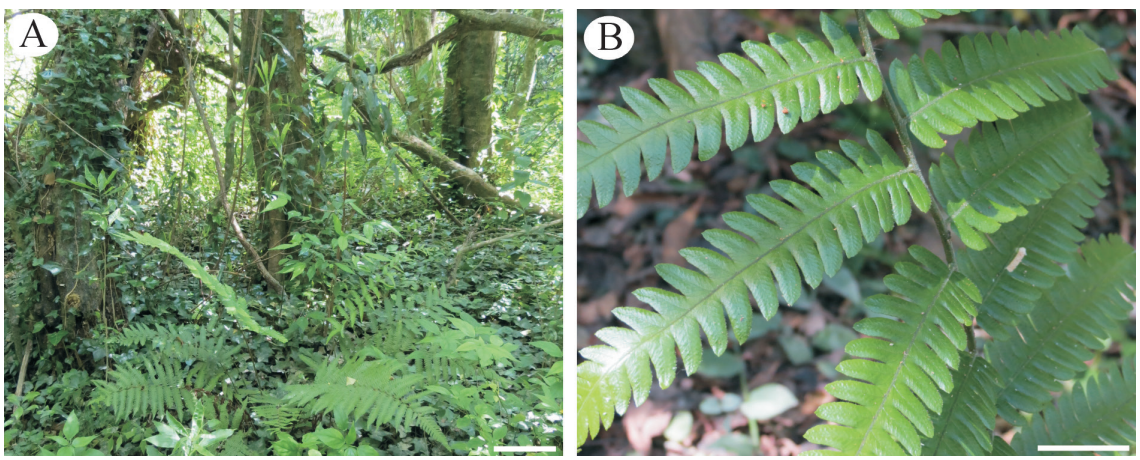

Fig. 1. A: Habitat of *Ctenitis submarginalis* in Punta Lara Natural Reserve. B: Detail of the lamina. Scale bars: A: 15 cm, B: 3 cm.

For obtain the spores, portions of fronds were keeping in paper bags and exposed to a light heat of 40 watts for a period of 72 hours. Later, the spores were separated with metallic mesh of 44 and 88 µm of diameter.

Before sowing the spores, they were disinfected with a hypochlorite sodium solution of 10% (v/v) during 3 minutes and later washed in distilled water.

The spores were sowing in Dyer (1979) medium solidified with agar (10 g/L) in petri dishes of 9 cm of diameter, previously sterilizer in autoclave (20 min., 1 atm., 120 ºC). The dishes were maintained in culture chamber at 22 ± 2 ºC and 12 hours of light (15 w/54).

The cultures were studied two or three times a week. Percentage germination was estimated on a random sample of 50 spores for each petri dish until the maximum germination was reached.

The observations and photographs were made under a Nikon Labophot-2, Nikon Eclipse E 200 microscopes and a Nikon SMZ 1000 stereoscopic microscope.

Results

Spores

The spores are monolete, light brown and elliptic in polar view. In equatorial view, the proximal face is convex and the distal face is hemispheric. The equatorial diameter is 37 (40) 45 µm, and the polar diameter is 28 (31) 35 µm. The laesurae are 5 (6) 9 µm long.

The surface is folded, with irregular folds in form and size (Fig. 2 A).

Gametophyte

Spores germinated six day after sowing. The percentage of germination was 78 % eight days after sowing but at fifteen days the percentage increase at 88 %.

The first step of the germination was the emergence of a hyaline, unicellular rhizoid (Fig. 2 B). Then, the chlorophyllous prothallial cell undergoes divisions to generate a short, uniseriate filament of 3-6 cells long with abundant chloroplast (Fig. 2 C). Twenty-two days after sowing, divisions in the apical cells of the filament take place and a hair located on a tip of the prothallus can be observed (Fig. 2 D).

A continuous meristematic activity originates an incipient cordate gametophyte with numerous hairs (Fig. 2 E). These hairs are chlorophyllous, unicellular, glandular and are distributed on the protallus margin (Fig. 2 F). The typical cordate gametophyte, with a deep notch and symmetric wings appears at 50 days (Fig. 2 G, 3 A).

The gametophyte of *Ctenitis submarginalis* produce gametangia 50 days after sowing. The sex organs are developed in the ventral side of separate gametophytes at the same time. The archegonia have curved necks with 6-8 cells, appear near the apical notch and some glandular hairs are observed between them (Fig. 2 H). The antheridia are located in the rizoids area and mixed with them. They are globoids and are composed by three cells: basal, annular and opercular (Fig. 2 I). Chloroplasts in the antheridial cells can be observed.

Female gametophytes were bigger than the male ones and more abundant in our cultures.

Sporophyte

The sporophytes emerged 190 days after sowing. The first sporophyte leaf could be bi, tri or tetra lobate, with open dichotomous type of venation (Fig. 3. B, C, D). Two different kinds of hairs were found on the lamina: 1) unicellular glandular hairs, similar to the ones of the prothallus, is developed in the margin and on both sides of the lamina (Fig. 3 D), 2) filiform hairs up to 3 cells long, with obtuse apical cell and distributed on the veins (Fig. 3 E, F).

After the second leaf appears the sporophytes were transplanted to plastic cup with organic commercial soil and maintained with constant high humidity (Fig. 4 A, B). Then the plants were gradually acclimated and when the plants reached approx. 15 cm tall, they were transplanted to Punta Lara Natural Reserve for reinforcement the natural populations of *Ctenitis submarginalis* (Fig 4 C, D).

Discussion and Conclusion

Respect to the different types of germination proposed by Nayar & Kaur (1971), *Ctenitis submarginalis* correspond to the most common type of germination pattern in the leptosporangiate ferns, the *Vittaria* type. In the same way, the prothallial development of this species is the *Aspidium* type (Nayar & Kaur, 1969) in which in early stages of the gametophyte development a glandular hair can be observed on a tip of the prothallus. The same type of

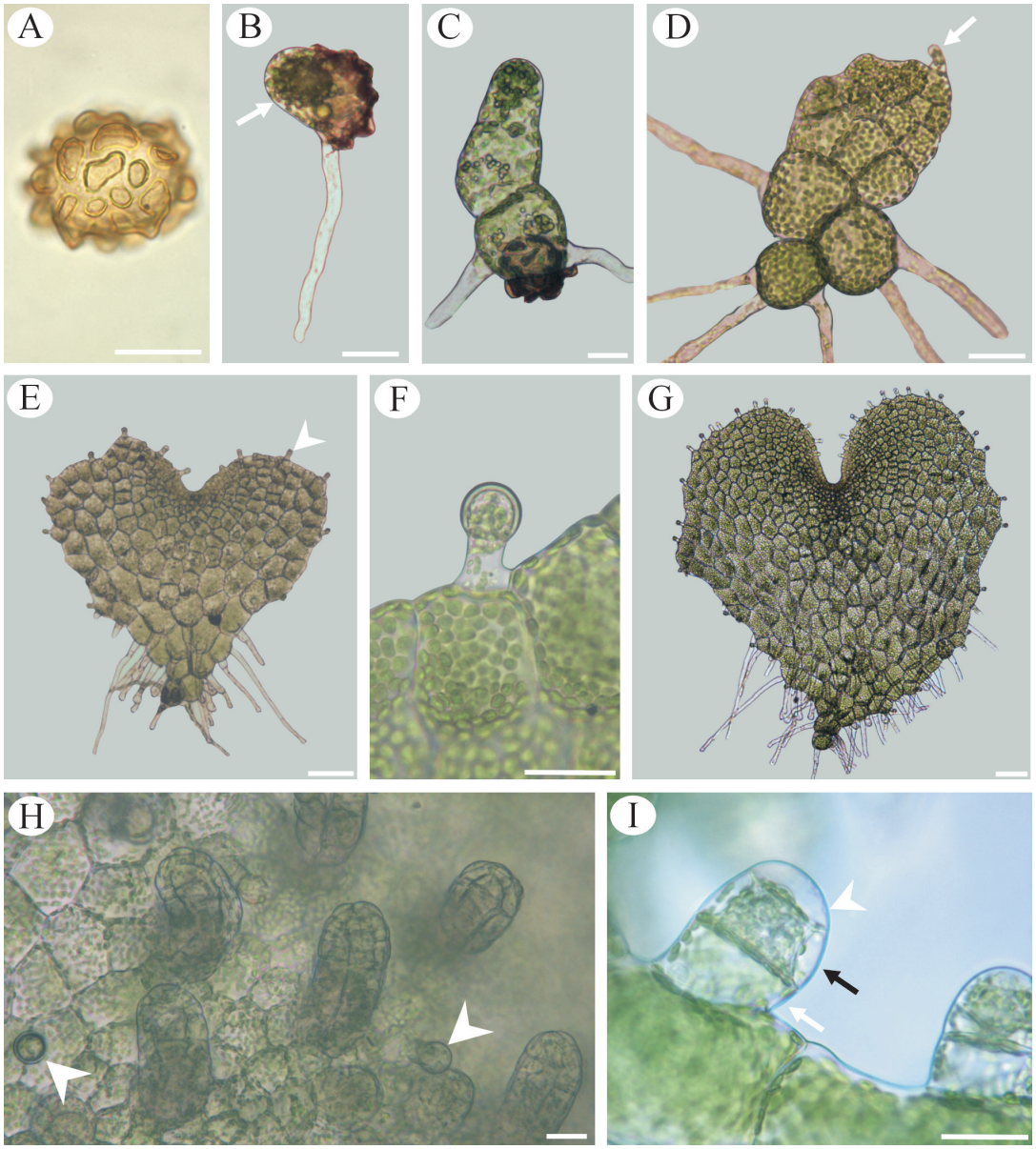

Fig. 2. Spores and gametophytes of *Ctenitis submarginalis*. A: Monolete spore with folded perispore. B: Germinated spore with a rhizoid and a prothallial cell (arrow). C: Short uniseriate filament of 3 cells. D: Hair located on a tip of the protallus can be observed. E: Young cordate gametophyte with glandular hairs on the protallus margin. F: Detail of a glandular hair. G: Cordate gametophyte with well developed and symmetric wings. H: Arquegonia with some glandular hairs (arrowheads) between them. I: Antheridium composed by a basal (white arrow), anular (black arrow) and opercular cell (arrowhead). Scale bars: A-C: 20 µm, D: 50 µm, E: 100 µm, F: 20 µm, G: 100 µm, H, I: 20 µm.

spore germination and gametophytic development were cited by Kaur & Devi (1976) and Meng *et al.* (2008) in other species of *Ctenitis*.

At the present, only a few species of *Ctenitis* were studied, therefore more investigations in other species of the genus would be necessary to clarify if the gametophyte development will be a useful character for systematic or phylogenetic purposes.

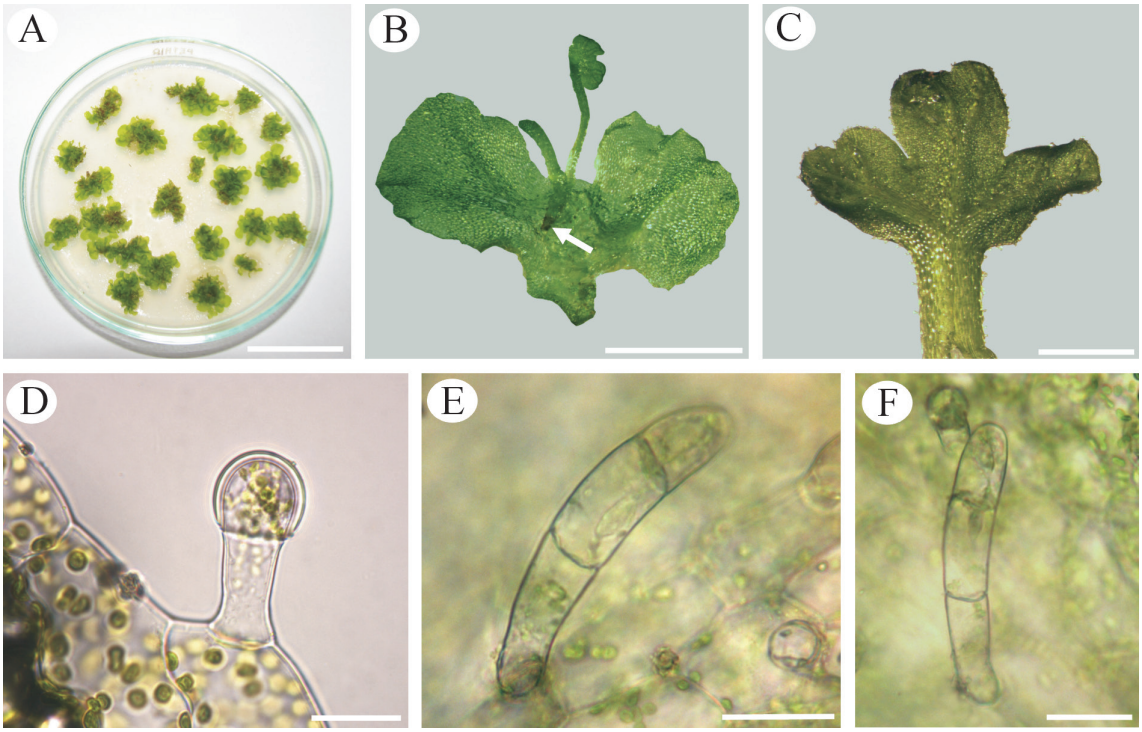

Fig. 3. Gametophytes and young sporophytes. A: Culture of mature gametophytes in Dyer medium. B: Gametophyte and sporophyte with first and second leaves. The small root of the sporophyte can be observed (arrow). C: Trilobate sporophyte leaf. D: Glandular hair of the lamina. E, F: Filiform hairs with obtuse apical cell. Scale bars: A: 3 cm, B: 2,5 mm, C: 1 mm, D: 25 µm, E-F: 50 µm.

The adult sporophyte of the genus *Ctenitis* has distinctive joint hairs on the leaves. These hairs have short cells, reddish joints and often are called as ctenitoid hair. This character is used to separate *Ctenitis* from other genera as *Dryopteris* and *Thelypteris,* with which *Ctenitis* has been confused and combined for long time (Mickel & Smith, 2004). In our cultures, the first sporophyte leaves were covered with a lot of glandular hairs and some multicellular hairs on the veins. These multicellular hairs correspond to the characteristic ctenitoid hairs and with our results we can confirm that this kind of hairs appears in early stages of the sporophyte development. Likewise, the glandular hairs that appear in gametophytes are very similar in shape and size to those present in the first sporophyte leaves.

The spores of the species studied have a high percentage of germination, about of 88 % fifteen days after sowing and gametangia with the typical morphology of the leptosporangiate ferns are observed in the mature gametophytes after 50 days.

Nevertheless, the first leaf of the sporophyte appears much later at 190 days. In the same way, Kaur & Devi (1976), when studied the gametophyte development of *Ctenitis ampla* and *C. recedens* Copel. reported that in their cultures fertilization takes places 5-6 months after sowing and young sporophytes are soon formed.

In our cultures, a lot of sporophytes grew from each petri dish. The small sporophytes obtained from in-vitro cultures have a high percentage of survival after two or three transplant to the plastic cups and many adults plants of *Ctenitis submarginalis* were produced. These results show that the in-vitro spore germination is an efficient propagation technique to obtain the plants that reinforce the small populations which grow in the natural area, reducing the risk of local extinction of the species.

Small quantity of spores is required for obtained numerous plants on in-vitro cultures of *Ctenitis*, therefore this method have a low impact in the

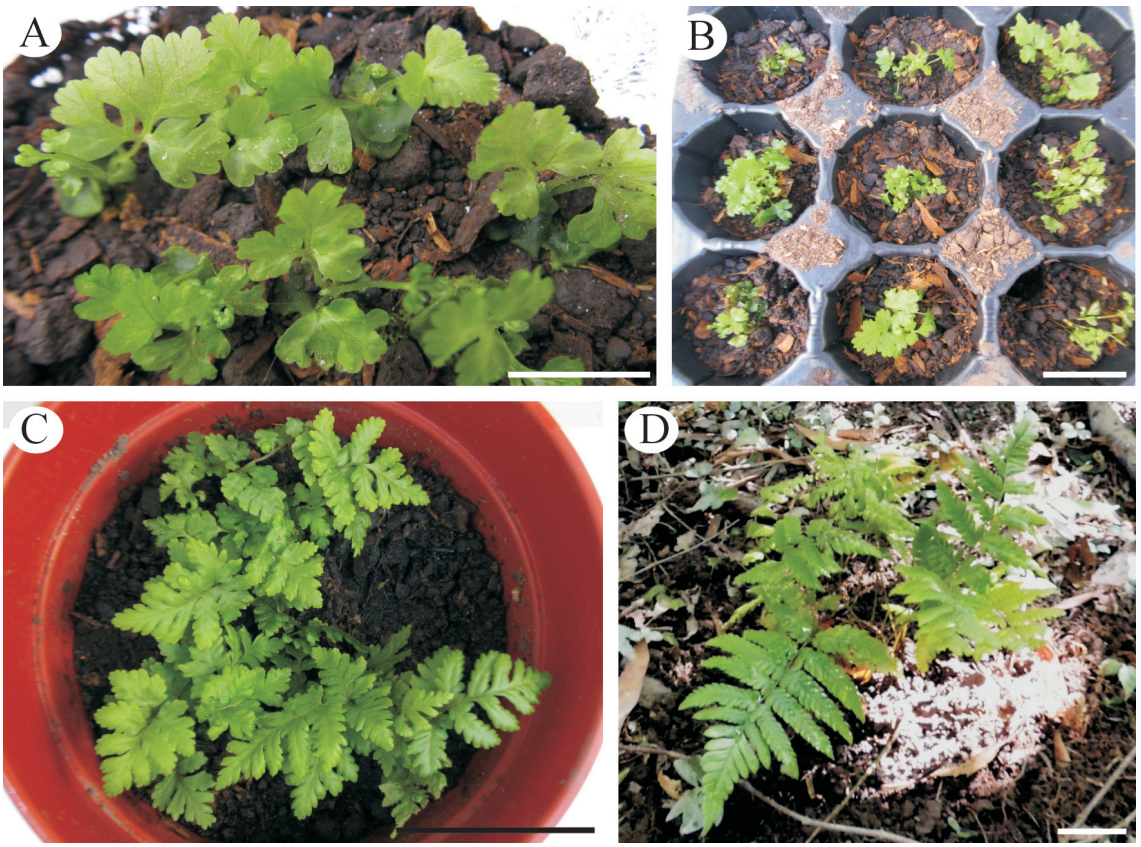

Fig. 4. A, B: Young sporophytes growing in plastic cups with high humidity. C. Plants in the period of acclimatization. D: Plant transplanted to Punta Lara Natural Reserve for reinforcement the natural populations of *Ctenitis submarginalis*. Scale bars: A: 1 cm, B: 2.5 cm, C-D: 5 cm.

wild populations of the studied fern. There are few specimens of this species in the reserve, so it is presumed that there are factors that would affect reproduction, especially spore germination and gametophytic development. Additionally, the in-vitro propagation of this species could be an effective method to obtain abundant individuals for ornamental purposes.

The plants transplanted to the reserve will be periodically monitoring to control the percentage of survival specimens, growth, time of fertile frond production and appearance of juvenile plants. Furthermore, other conservation action that implies *ex-situ* conservation will be developed for *Ctenitis submarginalis* from Punta Lara Natural Reserve. Today, ex situ measures are widely recognized as an effective tool for fern preservation, complementary to in situ conservation programmes (Ibars & Estrelles, 2012). In this way, the spores of this species are being cryopreserved and a successful method for long-term storage which retains the spore viability will be tested. These studies will contribute with the conservation of the species in Buenos Aires province and could be a first step to extend this program to other areas from Argentina.

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