Anatomical, Histochemical and Cytogenetic Features of *Doryopteris triphylla* (Pteridaceae)

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Abstract

Doryopteris triphylla (Pteridaceae-Cheilanthoideae) grows in xeric habitats in Brazil, Paraguay, Uruguay and Argentina. The aim of this study was to characterize *D. triphylla* anatomically, histochemically and cytogenetically. For anatomical characterization, rhizomes, roots, petioles and leaves were made and then stained using Safranine-Astra Blue for further observations. Leaf blades were also cleared. For histochemical analysis, leaf cross sections were stained with different reagents to identify glandular trichomes compounds. For cytogenetic characterization, a karyogram was performed using laboratory cultivated roots. Results show a dictyostelic rhizome covered with scales with apical secreting gland; diarch roots; petiole cross-sections show thick cuticle, uniseriate epidermis, parenchymatic cortex cells with thick walls and a vascular bundle with two xylem groups; and hypostomatic fronds with glandular trichomes. Histochemical studies of secretion products of the glandular trichomes were positive for polysaccharides, pectins, lipids, acid lipids, dihydroxyphenols, phenols and flavonoids. Cytogenetically, *D. triphylla* is described as a diploid species (2n = 60), with chromosomes gradually decreasing in size. The apical glands in scales of rhizomes, the presence of two xylem groups in the vascular bundle in the petiole and the glandular trichomes on the abaxial surface are new contributions to the species. The type of chemical products secreted by glandular leaf trichomes and karyotype estimation is shown for the first time in this species.

Keywords

Anatomy, Histochemistry, Karyogram, Pteridaceae

1. Introduction

Cheilanthoid ferns (Pteridaceae, Cheilanthoideae) are characterized as a cosmopolitan and diversified group inhabiting arid and semiarid environments. Morphological circumscription of the genera of this subfamily has been difficult, generating taxonomic controversies [1]. Works focusing on the genera *Adiantopsis* Fée [2], *Argyrochosma* (J. Sm.) Windham [3] [4], *Astrolepis* D.M. Benham & Windham [5] [6], *Doryopteris* J. Sm. [7], *Gaga* Pryer, F. W. Li & Windham [8], *Myriopteris* Fée [9], *Notholaena* R. Br. [10], and *Pellaea* Link [11], among others, indicate that the study and circumscription of the genera of the subfamily Chelanthoid is a priority when analyzing cheilanthoid ferns.

The genus *Doryopteris* s.s. was circumscribed by Yesilyurt *et al.* (2015) [7] with 33 species, and recently Schuettpelz *et al.* (2016) [12] established 21 species. *Doryopteris triphylla* (Lam.) Christ (**Figure 1(a)**) is a xeromorphic species exclusive to South America, distributed from southern Brazil, eastern Paraguay, and northeastern Argentina to Uruguay, and from the northwestern Argentine mountain region, through Cordoba mountains to Buenos Aires. The species occurs in the sierras Chaco forest and piedmont deciduous forest, sometimes forming dense populations with great development of rhizomes and roots [13].

General characteristics of the morphology and anatomy of the sporophyte have been described elsewhere [13] [14] [15]. In general, there are few records of histochemical studies in ferns, particularly in *Doryopteris*; Salatino and Prado (1998) [16] reported the presence of glycosylated flavonoids in *Doryopteris ornithopus* (Mett.) J. Sm.

Reports on the karyotype of *Doryopteris* are limited to chromosome counts. The two basic chromosome numbers in the family Pteridaceae are x = 29 and 30 [17]. Moran and Yatskievych (1995) [18] indicate a base number of x = 30 for *Doryopteris*; most of the species are diploid (n = 30, 2n = 60) and a great part of the cytogenetic analyses are based on gametophytic chromosome counts [19] [20]. The phenomenon of interspecific polyploidy in the genus was mentioned for *D. nobilis* L., with n = ca 60 for individuals from Paraguay [20] and in *D. palmata* L., with n = 60 for material from the Galapagos Islands [18]. Karyotype analyses were performed for other ferns, such as some species of *Acrostichum* L., *Lycopodium* L. and *Woodwardia* Sm. [21] [22] [23], and of *Polypodium* L. [24].

The aims of this study were to characterize the anatomical traits of *Doryopteris triphylla* associated with its xeromorphic condition, analyze the chemical compounds secreted by the glandular trichomes, determine the sporophytic chromosome number, and estimate the corresponding karyotype.

2. Materials and Methods

2.1. Plants Materials

Botanical material was obtained from LIL, MCNS and SI [25]; 16 specimens were studied (**Table 1**).

2.2. Anatomical Studies

To study the characteristics of the abaxial and adaxial epidermis of the leaf blades, as well as of the reflexed margin, the material was subjected to the clarification technique of Dizeo de Strittmatter [26] and Astra blue staining [27]. For anatomical studies, cross free hand sections of rhizome, root, petiole, leaf blade and rachis were made. Sections were bleached in 1:1 commercial sodium hypochlorite: water, rinsed with distilled water and stained with safranin-Astra blue. In all cases, slides were mounted in a water/glycerin solution (1:1). Cross sections of petiole were made at three levels: basal (next to the rhizome), intermediate, and apical (next to the leaf blade). Stoma types were determined using the classification of Van Cotthem (1970) [28]; stoma length and width were measured and stoma density was calculated as the number of stomata per mm^2. Length of glandular trichomes and paraphyses was measured, with 10 repetitions; mean and standard deviation (sd) were calculated. Mean thickness of cell wall of the rhizome scales was measured. The anatomical descriptions of the rhizome were based on Metcalfe and Chalk (1972) [29].

2.3. Histochemical Tests

For histochemical tests, cross sections of fresh fronds of *Doryopteris triphylla* were made and the following reagents were applied: Toluidine blue to detect polysaccharides [30], Nile Blue for neutral and acidic lipids [31], Ferrum chloride for dihydroxyphenols (catechol phenols) [32], Phloroglucinol stain for lignin [31], Neutral red for lipids [33], Ruthenium red for non-cellulose polysaccharides such as pectin [31], Sudan IV for lipids [31], Vanillin/H_2SO_4 for phenols

Table 1. Studied material's references.

Place	Vouchers
Argentina. Buenos Aires-La Cascada	Morrone, Guissani 6238 (SI)
Argentina. Entre Ríos-El Palmar	Morrone 5881(SI)
Argentina. Catamarca-Tintigasta	Prado s.n. (MCNS)
Argentina. Tucumán-Barrancas Coloradas	Venturi 807 (LIL)
Argentina. Tucumán-Barrancas del Río Salí	Schreiter 8877 (LIL)
Argentina. Tucumán-Huasa Pampa	Villa Carenzo, Vaca 2122 (LIL)
Argentina. Tucumán-Ciudad Universitaria	Villa Carenzo, Legname 1874 (LIL)
Argentina. Tucumán-Río Loro	Villa Carenzo 1540 (LIL)
Argentina. Tucumán-San Pedro de Colalao	Delgado, Ríos, Neira 915, 916, 917, 919, 920, 921 (LIL)
Argentina. Tucumán-Las Higueritas	Legname, Cuezzo 4635C (LIL)
Argentina. Tucumán-Río Las Juntas	Castillón 3521A, B (LIL)

[35], Vanillin/HCl [34] and Aluminum trichloride [34] under UV for flavonoids.

2.4. Cytogenetic Studies

For cytogenetic studies, roots of plants cultivated in the laboratory were used; they were pretreated with 0.002 M 8-hydroxyquinoline at 4°C for 24 h. They were fixed with Farmer solution (ethanol:acetic acid 3:1). They were rinsed with distilled water, then hydrolyzed in 1N HCl at 60°C for 20 minutes, then rinsed in distilled water again and finally mounted and squashed with a drop of 2% propionic hematoxylin. Counts were made using seven metaphase plates. The karyogram was performed on a metaphase plate with well dispersed chromosomes, which were classified using the nomenclature proposed by Levan *et al.* (1964) [36].

Photographs of observations were taken with an Olympus Q-color digital camera attached to an Olympus BX43 microscope, 7.1 MP Canon Powershot camera attached to a Zeiss Axiostar Plus microscope, Olympus-U-CMA D3 camera mounted on Olympus CX41 microscope, and a Nikon camera mounted on Nikon SMZ 800 stereoscopic microscope. Observations of fluorescent stained sections were made with an Olympus BX43. U-TVO. 5xc-3 epifluorescence microscope, using a UV 365 nm filter.

3. Results

3.1. Morphology and Anatomy

Rhizome. Short rhizome of 2 - 5 mm in diameter, dictyostelic; from outside to inside, it is composed of a single-layer epidermis, and cortex of sclerenchyma tissue; vascular bundles are surrounded by 2 - 3 layers of pericycle and endodermis with Casparian band in the radial walls (**Figure 1(b)**). Rhizome are covered with lanceolate to ovate scales (1.5 − 2.5 × 0.2 − 0.5 mm), bicolored, with dark sclerenchymatous central region and margins light-colored and erose. Scales exhibit a simple glandular cell at the apex (**Figure 1(c)**). Three types of cells were differentiated based on their wall thickness and position in the scale: central cells, of polygonal form and irregular wall thickenings (diameter = 8.42 μm, sd 0.99 μm); intermediate cells more irregularly shaped and less thickened (diameter = 4.58 μm, sd 0.85 μm); and marginal cells irregular to spindle-shaped and of thin walls (diameter = 2.44 μm, sd 0.55 μm) (**Figure 2(a)**).

Root. The root has a uniseriate epidermis composed of thin-walled cells; the cortex has 2 - 3 parenchyma layers of irregular and thin wall cells, and 4 - 6 layers of sclerenchyma cells of non-lignified thickened walls that are near the diarch stele (**Figure 2(b)**). The vascular cylinder is surrounded by endodermis with thickenings in the radial walls and two pericycle layers.

Petiole. In cross section, the petiole is terete to slightly semi-terete; it exhibits single-layered epidermis covered with a thick cuticle. The cortex has 2 - 3 layers of thick-walled parenchyma cells and 5 - 6-layers of thin-walled parenchyma cells. The vascular bundle is surrounded by 2 - 3 layers of pericycle and endodermis with thickened radial walls (**Figure 2(c)**); it is composed of two
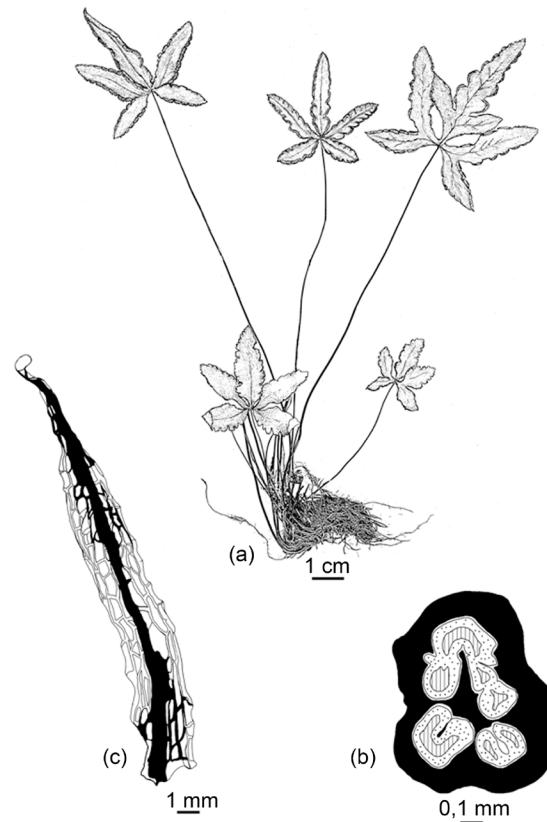
Figure 1. Sporophyte of *Doryopteris triphylla;* (a)—Plant; (b)—Cross section of rhizome; (c)—Rhizome scale; (d)—Detail of gland on the apex of rhizome scale; Scales: A = 1 cm; B = 1 mm; C = 0.1 mm; D = 1 μm.

xylem groups, the larger one oriented to the dorsal side, in an open V arrangement, and the other group, in ventral position, composed of 5 - 10 xylem elements, is observed at the three section levels.

Leaf blade. In the abaxial epidermis, cells are rectangular, with sinuous walls and smooth cuticle. Three types of stomata are observed: anomocytic, diacytic and polocytic (52%, 36% and 12%, respectively); mean length of stomata is 42.6 μm (sd 0.42 μm) and mean width is 36.1 μm (sd 3.95 μm). Stomata are evenly distributed and at the same level of or slightly above epidermis cells; the recorded density is 127 stomata/mm^2 (**Figure 2(d)**). The indument is composed of bicellular glandular trichomes, consisting of a unicellular foot and head 63.43 μm (sd 5.91 μm) long, and located in the abaxial epidermis (**Figure 2(e)**). Epidermis cells on the adaxial surface and reflexed margin exhibit sinuous and thick walls (**Figure 2(f) Figure 2(g)**).

In cross section, the leaf blade is dorsiventral and hypostomatic; both epidermis are single-layered and have a thick cuticle; the palisade parenchyma is composed of 1 - 2 cell layers occupying 1/3 of lamina thickness, whereas the spongy parenchyma with big intercellular spaces (**Figure 2(h)**), is 5 - 6-layered and occupies 2/3 of the lamina thickness; vascular bundle surrounded by a conspicuous endodermis with Caspary bands (**Figure 2(i)**). The pseudoindusium is subterminal, multilayered, with tracheids in the proximal portion, and two cell layers

in the recurved distal portion (**Figure 2(j)**).

In the sori, paraphyses are observed among sporangia; paraphyses are mostly originated in the receptacle, a few of them seem to originate from the base of the sporangium foot. These paraphyses are bicellular glandular trichomes composed of a foot and a head, 48.66 μm (sd 5.22 μm) in length (**Figure 2(j)**).

3.2. Histochemical Analysis

The secretion products of the glandular trichome are presented in **Table 2** and **Figure 3**.

3.3. Cytogenetic Studies

The results indicate a sporophytic chromosome number of 2n = 60. Chromosome length is 1.59 to 4.38 μm (**Figure 4**). Total length of the haploid chromosome complement is 100.06 μm. The estimated karyotype formula is 2 m + 7 sm+ 12 st + 9 t (**Figure 4**). Values of total length of each chromosome (c),
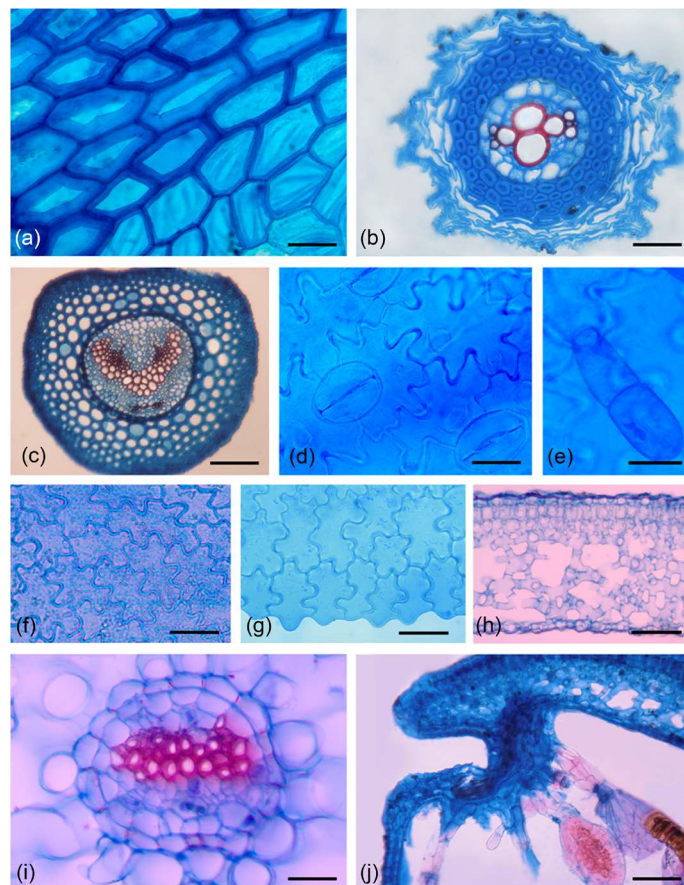

Figure 2. Anatomy of *Doryopteris triphylla*; (a)—Cells of rhizome scale; (b)—Cross section of root; (c)—Cross section of petiole; (d)—Paradermal view of abaxial surface; (e)—Detail of trichome on abaxial epidermis; (f)—Paradermal view of adaxial surface; (g)—Paradermal view of reflexed margin; (h)—Cross section of leaf blade at the mid-vein level; (i)—Detail of vascular bundle of leaf blade; (j)—Cross section of leaf blade at the level of reflexed margin. Scales: (a) = 20 μm; (b) = 180 μm; (c) = 160 μm; (d) = 30 μm; (e) = 40 μm; (f), (g) = 80 μm; (h) = 260 μm; (i) = 50 μm; (j) = 80 μm.

Table 2. Histochemical identification of compounds in glandular trichome of *Doryopteris triphylla.*

Reagent	Target compounds	Reaction	Figure
Toluidine Blue	Polysaccharides	+	3(a)
Ruthenium Red	Pectin	+	3(b)
Phloroglucinol	Lignin	-	3(c)
Sudan IV	Lipids	+	3(d)
Nile Blue	Acid lipids	+	3(e)
Neutral Red	Lipids	+	3(f)
Ferrum Chloride	Dihydroxyphenols	+	3(g)
Vanillin/H_2SO_4	Phenols	+	3(h)
Vanillin/HCl	Flavonoids	+	3(i)
Aluminum Chloride	Flavonoids	+	3(j)
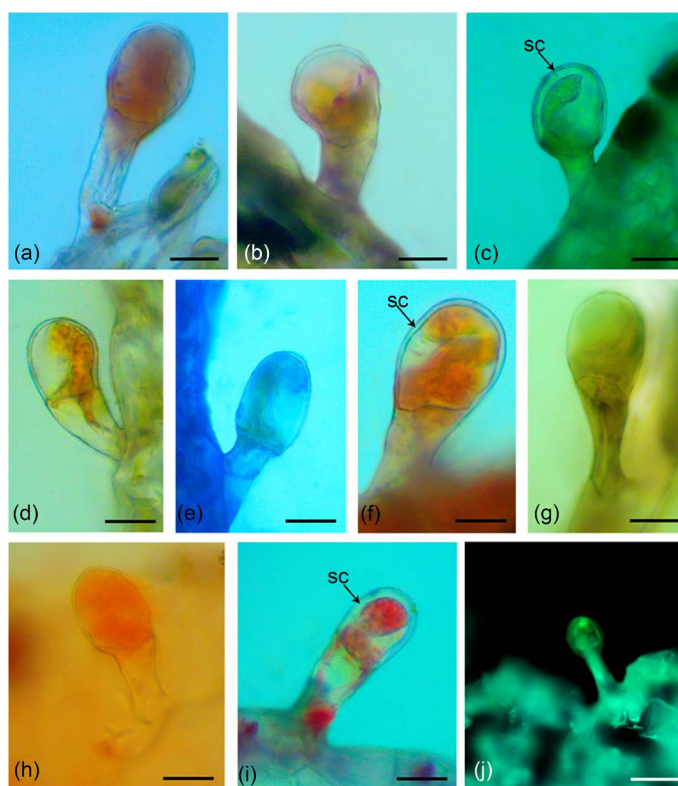
Figure 3. Histochemical analyses, detail of glandular trichomes of *Doryopteris triphylla.* (a)—Toluidine Blue; (b)—Ruthenium Red; (c)—Phloroglucinol; (d)—Sudan IV; (e)—Nile Blue; (f)—Neutral Red; (g)—Ferrum Chloride; (h)—Vanillin/H_2SO_2; (i)—Vanillin/HCl; (j)—Aluminum Chloride. sc = subcuticular chamber. Scales: (a), (b), (c), (e), (g) = 30 μm; (d), (h), (i) = 40 μm: F = 27 μm; (j) = 100 μm.

length of the short arm (s), length of the long arm (l) and centromeric index (ci) are shown in **Table 3**. The two pairs of metacentric (m) chromosomes are very different from each other, with one of the pairs being of very small length (1.59 μm) and the other, of intermediate length (3.14 μm). Submetacentric (sm) and
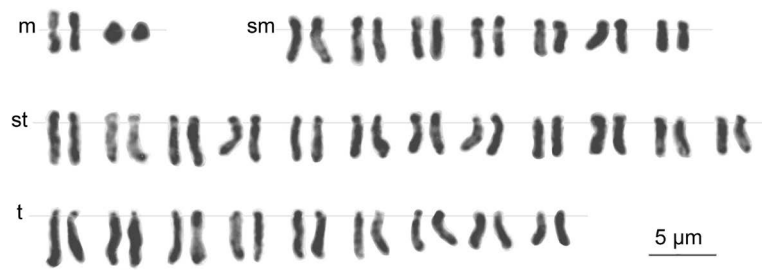
Figure 4. Karyogram of *Doryopteris triphylla.* Chromosome type: m = metacentric; sm = submetacentric; t = telocentric; st = subtelocentric. Scale = 5 µm.

Table 3. Cytogenetical analysis. Values of total length of each chromosome (C), length of the short arm (s), length of the long arm (l) and centromeric index (Ci) are shown.

Chromosome pair	C (µm)	l (µm)	s (µm)	Ci%	Chromosome type
1					
2	3.14	1.71	1.43	45.54	m
3	1.59	0.82	0.76	47.79	m
4	3.69	2.8	0.88	23.84	sm
5	3.43	2.7	0.73	21.28	sm
6	3.21	2.49	0.71	22,11	sm
7	3.15	2.21	0.94	29.84	sm
8	2.78	2.17	0.61	21.94	sm
9	2.65	1.98	0.67	25.28	sm
10	2.25	1.7	0.55	24.44	sm
11	3.26	2.74	0.53	16.25	st
12	4.04	3.45	0.59	14.60	st
13	3.96	3.28	0.68	17.17	st
14	3.9	3.35	0.55	14.10	st
15	3.64	3.07	0.52	14.28	st
16	3.62	3	0.62	17.12	st
17	3.33	2.75	0.57	17.11	st
18	3.21	2.79	0.42	13.08	st
19	3.16	2.62	0.54	17.08	st
20	3.11	2.62	0.49	15.75	st
21	3.08	2.63	0.44	14.28	st
22	2.94	2.54	0.4	13.60	st
23	4.38	3.86	0.52	11.87	t
24	4.22	3.94	0.27	6.39	t
25	4.04	3,53	0.5	12.37	t
26	3.87	3.5	0.36	9.30	t
27	3,78	3.33	0.45	11.90	t
28	3.5	3.16	0.34	9.71	t
29	2.95	2.65	0.3	10.16	t
30	2.73	2.5	0.1	3.66	t

subtelocentric (st) chromosomes vary in size, ranging between 2.25 - 3.43 μm and between 2.94 - 3.26-μm in length, respectively. Telocentric (t) chromosomes form two groups, one containing three pairs of chromosomes of length ranging between 3.5 - 4.38 μm, and the other including moderately smaller chromosomes (2.76 - 3.26 μm).

4. Discussion and Conclusions

The anatomy of the dictyostelic rhizome of *Doryopteris triphylla* does not contribute with traits for species identification. This structure has also been observed in other *Doryopteris* species [37], in other Pteridaceae species and in different families of ferns [38]. Rhizome scale with a glandular trichome on the apex is a morphological trait that has not been described in floristic studies mentioning this species [13] [15]. This trait may have been unnoticed because trichomes often fall off easily when scales are adult, as observed in some species of *Pteris*: *P. ciliaris* Eat.*, P. cretica* L.*, P. denticulata* Sw., *P. ensiformis* Burm. f. and *P. multifida* Poir. [39].

The root structure is similar to that of other cheilanthoid ferns, *Doryopteris concolor* (Langsd. & Fisch.) Kuhn*, D. lorentzii* (Hieron.) Diels and *Trachypteris pinnata* (Hook. f.) C. Chr. [37] [40] [41], both genera regarded as very closely related [42].

The petiole of *D. triphylla* exhibited the presence of an additional group of xylem elements in the vascular bundles, which has been also observed in others *Doryopteris*, such as *D. concolor* and *D. lorentzii* [37] [40]. Similar findings were reported for the subfamily Cheilanthoideae, *Cheilanthes arequipensis* (Maxon) R. M. Tryon & A. F. Tryon*, C. buchtienii (Rosenst.)* R. M. Tryon, *C. obducta* Mett. ex Kuhn*, C. volcanensis* de la Sota and *Myriopteris aurea* (Poir.) Grusz & Windham (Hernández, pers. comm.).

In the epidermis of *D. triphylla*, stomata length, width and density have mean values similar to those reported for other cheilantoid ferns: *Adiantopsis chlorophylla* (Sw.) Fée, *Argyrochosma nivea* (Poir.) Windham, *Myriopteris aurea* (Poir.) Grusz & Windham, *Cheilanthes buchtienii* (Rosenst.) R. M. Tryon, *Cheilanthes notholaenoides* (Desv.) Maxon ex Weath., *Cheilanthes pilosa* Goldm., *Doryopteris concolor* and *D. lorentzii* [41]. Hevly (1963) [43] and Tejero Diez (2009) [44] consider that ferns from xeric environments present a tendency to reduction in size and stoma density; in *D. triphylla*, the obtained values agree with ecological characteristics of a species inhabiting seasonally dry environments.

In cross section of the leaf blade, the thickness of the palisade parenchyma is markedly lower than that of the spongy parenchyma; this trait was indicated for other Pteridaceae species occurring in exposed sites, such as *Adiantopsis chlorophylla, D. concolor, D. lorentzii* and *Trachypteris pinnata* [37] [40] [41] [45]. By contrast, Hevly (1963) [43] and Tejero Diez (2009) [44] state that an increase in palisade parenchyma and a decrease in spongy parenchyma are adaptations of xeromorphic ferns.

In sori, glandular paraphyses occurring with sporangia are mentioned for the first time in this species. These sterile structures differ from the trichomes on the abaxial side of the lamina on being of larger size. Thus, we agree with Wagner (1964) [46] that paraphyses tend to provide some sort of protection to the developing receptacle and young sporangia from external effects, and that they are usually found in ferns of sunny, dry or exposed environments as is the case of *Doryopteris triphylla*.

Histochemical tests allowed us to detect and locate *in situ* the principal metabolites present in trichome secretions. Our results indicate that the content is of complex nature, including polysaccharides, lipids, phenols and flavonoids. The presence of non-cellulosic polysaccharides such as pectin was demonstrated using Rutheniun red in the cell wall of the secreting gland and in the site of attachment of the stalk with the lamina. Pectin might be related to translocations of secondary metabolites [47].

Positive reactions for lipids were obtained in the glandular head using Sudan IV and Neutral red, and for acid lipids in all the trichome with Nile blue. According to Werker (2000) [48], lipid metabolites would play a protective role.

Phenolic substances, detected using ferric chloride and Vanillin/H_2SO_4, were found in trichomes. Flavonoids were the only type of phenolic compounds histochemically identified in these trichomes using Vanillin/HCl and aluminum chloride. Those compounds are present in glandular trichome secretions in numerous species [49] and are important for plant protection against visible and ultraviolet light, and play a significant role in plant chemical defense against the attack of herbivores, bacteria and fungi [50] [51]. The histochemical analysis of glandular trichomes shows a subcuticular chamber, which is interpreted as the site of accumulation of chemical substances before their release.

The revision of cytogenetic records in the literature reveals a noticeable lack of works aimed at establishing the inter- and intra-chromosomal relationships that provide the parameters of a karyotype. The comparison of the karyotype estimated for *D. triphylla* with the few karyotypes described for other fern genera shows some similarities. The karyotype formula of *D. triphylla* exhibits metacentric and submetacentric chromosomes, a characteristic shared with others species of *Acrostichum* [21], *Lycopodium* [22] and *Woodwardia* [23]. Likewise, *D. triphylla* is diploid and shows a karyogram whose chromosome length (1.59 μm to 4.38 μm) is very similar to that observed in diploid species of *Polypodium* (2.2 μm to 4.5 μm) [24]. The comparison also shows that the length of the haploid chromosome complement of *D. triphylla* is similar to the length observed in diploids of *Woodwardia* (ca. 100 μm) [23]. However, chromosome length of *D. triphylla* and *Polypodium* is similar, but in *Polypodium*, most of the chromosomes are telocentric and the remaining chromosomes of the complement are acrocentric. Murray (1985) [24] and Marcon *et al.* (2003) [21] did not mention metacentric or submetacentric chromosomes for that genus.

Moreover, although *D. triphylla* and the *Acrostichum* species analyzed by Marcon *et al.* (2003) [21] are diploid, with 2n = 60, and similar in terms of

chromosome morphology, chromosome length of *Acrostichum* (approximately 5.0 μm to 8.0 μm) is almost twice that observed in *D. triphylla*, which is reflected in its haploid chromosome complement, of nearly 100 μm in *D. triphylla* and 192 μm in *Acrostichum*.

We conclude that *Doryopteris triphylla* is a typically xeromorphic fern, since it exhibits sclerenchyma tissue in root, rhizome and petiole, glandular trichomes in frond, sinuous thickened walls in rectangular epidermis cells; thick cuticle and rhizome scales with glands. All of these traits were indicated by Hevly (1963) [43] for ferns occurring in xeric habitats. Anatomical characters, trichome secretion products, chromosome counts that confirm the basic number established for the genus (x = 30) and karyogram contribute with novel information for *Doryopteris*, which may help understand the phylogenetic relationships within the genus.

Acknowledgements

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