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Ferns and Lycophytes as new challenges

Anatomical studies of Brazilian Amazonian *Isoëtes* species: inferences on habitat adaptation

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Abstract

Isoëtes is a cosmopolitan genus of aquatic lycophytes, containing more than 200 species. In Brazil, the genus comprises 29 species, with three occurring in Pará state, Amazon. *Isoëtes cangae* and *I. serracarajensis* are endemic to the ferruginous outcrops of Serra dos Carajás, and *I. amazonica* occurs on the inundated shores of the Tapajós River. Despite the great diversity of quillworts in South America, their anatomy remains unknown. This study discusses Brazilian Amazon species' leaf and root anatomical traits in relation to habitat and genetic diversity. The amphibious *I. amazonica* and *I. serracarajensis* were observed to have similar stomata and cuticular ornamentations. *Isoëtes cangae*, a fully aquatic species, had smaller epidermal cells and a smooth cuticle and showed slight differences regarding the lacuna diaphragm. The genetically closer species from Carajás both lacked peripheral fiber strands on the leaves. Our study complements current knowledge regarding the morphoanatomy of Amazonian species and provides a better understanding of their biology, contributing to the development of conservation strategies for these species.

Key words: anatomical traits, aquatic plants, peripheral fibers, Serra dos Carajás, stomata.

Resumo

Isoëtes é um gênero cosmopolita de licófitas aquáticas, contendo mais de 200 espécies. No Brasil, o gênero compreende 29 espécies, e três delas ocorrem no estado do Pará, Amazônia. *Isoëtes cangae* e *I. serracarajensis* são endêmicas dos campos rupestres ferruginosos da Serra dos Carajás, e *I. amazonica* ocorre nas planícies de inundação do rio Tapajós. Apesar de sua grande diversidade na América do Sul, a anatomia dessas espécies ainda é desconhecida. Este estudo discute sobre os caracteres anatômicos das folhas e raízes das espécies de *Isoëtes* da Amazônia Brasileira, relacionando-os ao habitat e à sua diversidade genética. As espécies anfíbias *I. amazonica* e *I. serracarajensis* apresentaram estômatos e ornamentações cuticulares semelhantes. *Isoëtes cangae*, uma espécie aquática, apresentou células epidérmicas menores, cutícula lisa e demonstrou diferenças relacionadas aos diafragmas das lacunas. As espécies de Carajás, geneticamente mais próximas, não apresentaram feixes de fibras periféricas nas folhas. Nosso estudo complementa o conhecimento atual da morfoanatomia das espécies Amazônicas, e contribui com a melhor compreensão de sua biologia, subsidiando o desenvolvimento de estratégias de conservação para essas espécies.

Palavras-chave: caracteres anatômicos, plantas aquáticas, fibras periféricas, Serra dos Carajás, estômatos.

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Introduction

Isoëtes L. is a genus of heterosporous lycopsids distributed worldwide and comprises approximately 250 species (Pereira *et al.* 2017). The genus is the only remaining representative of the Isoetales, occupying a unique position in plant evolution (DiMichele & Bateman 1996; Pigg 2001; Yang & Liu 2015). These arborescent ancestors were key components of the coal-forming environments in the Carboniferous period (Phillips & DiMichele 1990; Hetherington *et al.* 2016). *Isoëtes* differ from other extant lycopsids in growth habit: sunken adaxial sporangia, sporangial trabeculae, leaves containing four air chambers, and a ligule with a basal glossopodium (Gifford & Foster 1989).

The genus retains many ancestral morphological and anatomical features (Pigg 2001). It has a reduced stem with determinate growth and secondary thickening and stigmarian roots that resemble those of rhizomorphic lycopsids (DiMichele & Bateman 1996). The elongated cylindrical leaves, typical in aquatic plants, contain air chambers (aerenchyma lacunae) that allow gas transport between buried and photosynthetic organs (Green 2010). The aerenchymatous tissue system indicates the presence of aquatic crassulacean acid metabolism (CAM) photosynthesis in arborescent lycopsids (Green 2010). CAM metabolism in modern *Isoëtes* species is adaptive for low underwater CO_2 availability (Keeley & Bowes 1982; Wickell *et al.* 2021).

Throughout *Isoëtes* evolution, repeated transitions between aquatic and terrestrial habitats are hypothesized to have occurred (Taylor & Hickey 1992). The extant species inhabit fully submerged or seasonal ponds, although a few terrestrial species also exist (Hickey 1986). Stomata are commonly seen in terrestrial taxa and amphibian taxa with emergent leaves (Prada 1979), although some aquatic taxa may also show stomata (*e.g.*, *Isoëtes howelii* Engelmann; Keeley 1981) (Prada & Rolleri 2003). However, some terrestrial and amphibious species may fail to produce functional stomata (Keeley 1983; Yang & Liu 2015). Epidermal cuticle ornamentations and subepidermal fibers are also associated with *Isoëtes* species habitats (Prada 1979; Prada & Rolleri 2003).

Previous studies showed that several anatomical traits of *Isoëtes* leaves, such as the number of interstellar canals, the shape of the translacunar diaphragm cells, the presence of intercellular pectic protuberances, peripheral fibrous strands, stomata, cuticular ornamentations, and the sporangium epidermis coloration, have diagnostic value (Pfeiffer 1922; Hall 1971; Prada 1979, 1983; Takamiya *et al.* 1997; Kott & Britton 1985; Hickey 1986; Taylor & Hickey 1992). Fluorescence and scanning microscopy techniques were used to document and categorize the endoderm of the interstellar canals and the aerenchyma lacunae (Romeo *et al.* 2000; Rolleri & Prada 2004) and the intercellular pectic protuberances (Prada & Rolleri 2003; Rolleri & Prada 2005). In addition to qualitative traits, micro-morphometric leaf parameters have also been identified as taxonomically important (Budke *et al.* 2005; Rolleri & Prada 2007). The diagnostic value of anatomical leaf characters was reviewed by Rolleri & Prada (2007). However, there is a paucity of literature on the root anatomy of *Isoëtes* due to its conserved form.

South America is one of the main centers of taxonomic diversity of *Isoëtes*, with an estimated 64 extant species (Troia *et al.* 2016). In Brazil, the genus comprises 29 species, three occurring in Pará state (Pereira *et al.* 2023). Phylogenetic studies have placed Brazilian *Isoëtes* into two distinct groups: the American and Gondwanan clades (Pereira *et al.* 2017). Although anatomical studies on representatives of the American clade have been carried out for several species, most from North America, no studies have been performed on representatives of the Gondwanan clade, which includes the Amazonian species *I. cangae* J.B.S. Pereira, Salino & Stützel, *I. serracarajensis* J.B.S. Pereira, Salino & Stützel, and *I. amazonica* A. Braun. This anatomical knowledge gap hampers our understanding of the anatomical diversity in the genus.

Isoëtes cangae and *I. serracarajensis* are endemic to the ferruginous outcrops of Serra dos Carajás and were described in the last decade (Pereira *et al.* 2016). *Isoëtes cangae* lives submerged in a permanent lake, whereas *I. serracarajensis* occurs in several seasonal ponds in Serra Norte, Serra Sul, Serra do Tarzan and Serra da Bocaina (Pereira *et al.* 2017; Nunes *et al.* 2018). *Isoëtes cangae* is diploid (2n = 22) and is the most likely maternal progenitor of the allotetraploid *I. serracarajensis* (Pereira *et al.* 2021). In addition to the morphological characters used in the species description (*e.g.*, megaspore ornamentation; Pereira *et al.* 2016), the presence of stomata and the size of the sporangium epidermal cells were also shown to be consistent in differentiating the two species (Cavalheiro-Filho *et al.* 2021). However, a deeper anatomical investigation was not performed on *I. cangae* and *I. serracarajensis*. The third species, *I. amazonica*, was collected in 1850 and described as the first quillwort from the Brazilian Amazon, being rediscovered in 2017 at its type locality on the inundated shores of the Tapajós River, Pará state (Pereira *et al*. 2019). *Isoëtes amazonica* is a tetraploid species and has a greater genetic distance from *I. cangae* and *I. serracarajensis* than the species of Carajás by each other (Pereira *et al*. 2021).

In addition to their restricted distributions, *I. cangae* and *I. serracarajensis* are also found in iron-rich substrates in areas subjected to mining activities, which may lead to habitat deterioration (Pereira *et al*. 2016). Serra dos Carajás contains the largest deposits of high-grade iron ores in the world, representing an important Brazilian mining complex (Lindenmayer *et al.* 2001). For this reason, *I. cangae* is classified as critically endangered (CR) and *I. serracarajensis* as vulnerable (VU) by the International Union for Conservation of Nature (IUCN 2023). *Isoëtes amazonica* is known from a single locality and is susceptible to the effects of human activities related to cattle farming, although it could potentially occur in other areas of the Amazon Basin; due to the lack of knowledge about its distribution range, *I. amazonica* is classified as data deficient (DD) according to IUCN criteria (IUCN 2023; Pereira *et al*. 2019).

Considering that (1) *I. amazonica* and *I. cangae* are currently known from single localities, (2) *I. amazonica* and *I. serracarajensis* occur in temporary, inundated habitats, (3) *I. cangae* and *I. serracarajensis* are endemic to iron-rich substrates interest for iron ore mining, (4) the occurrence region is undergoing significant changes in the hydro-climatological cycle because of the conversion of forest areas into grasslands (Souza-Filho *et al*. 2016), and (5) there is a lack of information regarding the morphoanatomical adaptations of these species to their habitats, we aimed to determine the anatomical variation of the leaves and roots of these Amazonian *Isoëtes* species that might be related to the habitats in which they occur.

Materials and Methods

Plant samples

Specimens of *Isoëtes amazonica* and *I. cangae* were each collected from a single locality in western and southeastern Pará state, respectively, whereas *I. serracarajensis* specimens were sampled from four temporarily flooded areas in Serra dos Carajás, southeastern Pará (Tab. 1). *Isoëtes amazonica* is subjected to annual seasonality. The rainy season ranges from December to July, and the dry season, when the species' habitat may be completely dry, ranges from August to November (Pereira *et al*. 2019). Similarly, *I. cangae* and *I. serracarajensis* are subjected to unimodal pluviometric cycles. The rainy season lasts from October to May, and the dry season from June to September (Viana *et al*. 2016). During the dry season, the inundated ponds may completely dry out.

Specimens of *I. amazonica* were collected in July 2017 (dry season) from a marsh area 2.5 km from the left bank of the Tapajós River, Santarém County (Fig. 1). The plants were found as terrestrials in wet soil. *Isoëtes cangae* specimens were obtained in February 2018 (rainy season) from the bottom of Amendoim Lake, Canaã dos Carajás County, in the South Range of Serra dos Carajás (Fig. 1). The specimens were submerged at a depth of 3.5 m. *Isoëtes serracarajensis* specimens were sampled from two temporarily flooded areas located in the North Range of Serra dos Carajás (N3 and N6 flooded areas, Parauapebas County) and two located in the South Range (ISV and S11 flooded areas, Canaã dos Carajás County) (Fig. 1). In February 2018 (rainy season), completely submerged plants were collected in the N3, N6, and ISV flooded areas at a maximum depth of 0.8 m for N3. Plants from S11 were sampled in June 2018 (dry season) at a depth of 0.1 m, with leaves partially covered by water (Tab. 1).

Plants of *I. cangae* (N = 10) and *I. serracarajensis* (N = 10/population) collected *in situ* were kept in pots filled with water and transported to the Instituto de Biodiversidade e Sustentabilidade - NUPEM (Universidade Federal do Rio de Janeiro). Some of these plants collected *in situ* were also grown in a greenhouse for complementary anatomical studies. Plants of *I. cangae* and *I. serracarajensis* were cultivated under complete and partial submergence, respectively. Specimens of *I. amazonica* (N = 5) were previously maintained in a growth chamber under partial submergence at Instituto Tecnológico Vale Diversidade e Sustentabilidade (ITV DS) and then transported to NUPEM. Four individuals of each species/population were used for the anatomical analyses.

Specimens from each locality were also pressed and dried at 70 °C for voucher preparation (Tab. 1). Permission to collect *I. amazonica*, *I. cangae*, and *I. serracarajensis* specimens was granted by the Chico Mendes Institute of Biodiversity of the Ministry of the Environment (ICMBio/MMA; license numbers 35897, 64187, and 59724, respectively).

Anatomical analyses

Light microscopy

Samples of the middle portion of the leaf and the basal portion of roots (up to 2 cm from the corm) were obtained from plants of *I. amazonica*, *I. cangae*,

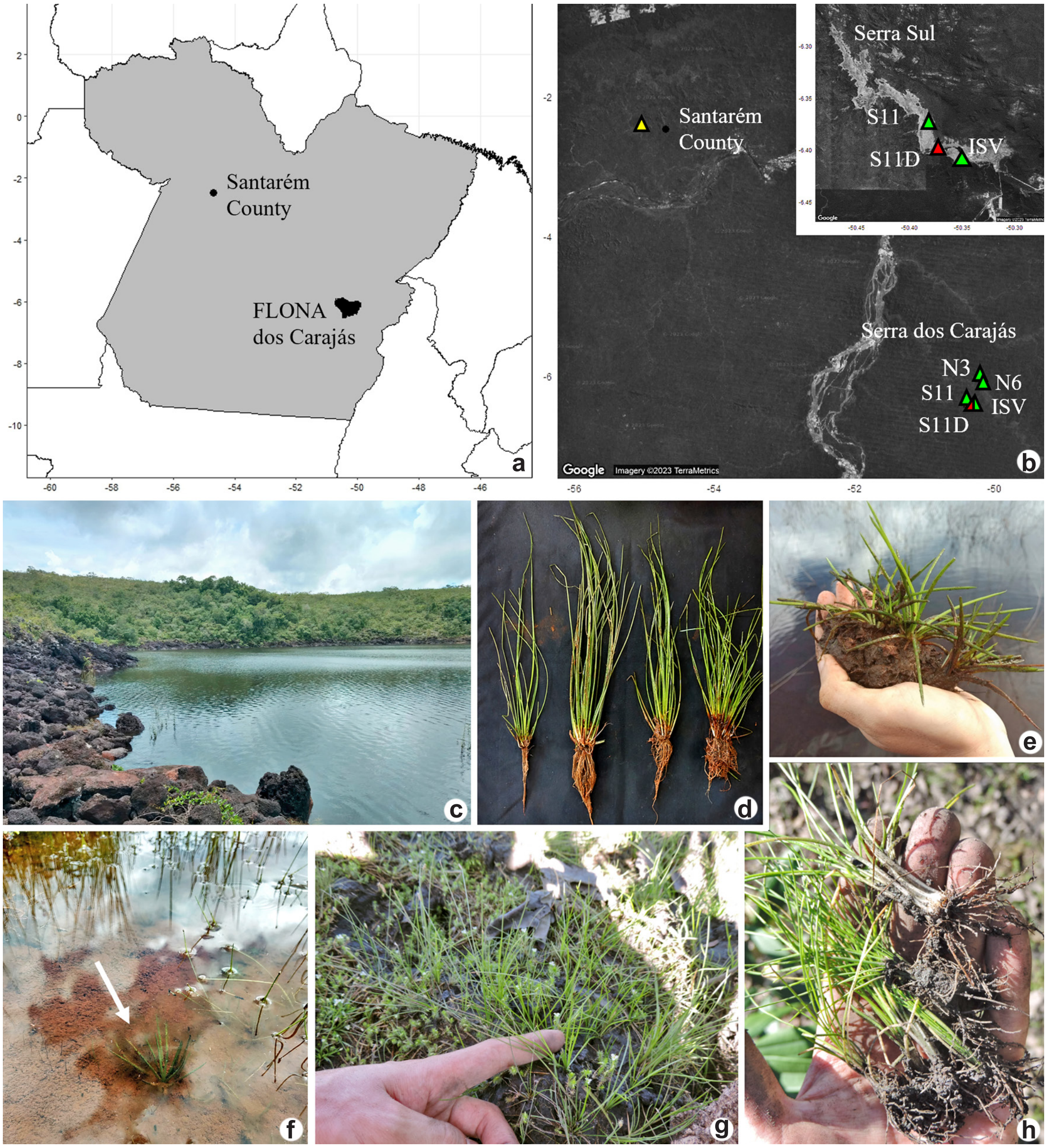

Figure 1 – a-h. Occurrence sites of the *Isoëtes* species found in Pará state, Amazon Basin – a. map of Pará state, indicating the location of Santarém and Floresta Nacional dos Carajás (FLONA dos Carajás); b. approximate map of Pará state, indicating the location where *Isoëtes amazonica* specimens were collected near Santarém (yellow triangle) and the points where *Isoëtes cangae* (red triangle) and *I. serracarajensis* (green triangles) from N3, N6 (Serra Norte), S11 and ISV (Serra Sul) were collected in FLONA dos Carajás; c. Amendoim Lake, the single occurrence location of *I. cangae*; d. adult specimens of *I. cangae* collected in February 2018; e. adult specimens of *I. serracarajensis* from the ISV flooded area collected *in situ*; f. adult specimen of *I. serracarajensis* from the N6 flooded area growing *in situ* (arrow); g. terrestrial specimens of *I. amazonica* growing *in situ*; h. adult specimens of *I. amazonica* collected *in situ*.

Table 1 – Voucher information.

Species	**Locality**	**Coordinates**	**Specimen**
Isoëtes amazonica	Marsh area nearby Tapajós River	02°24'15.15"S, 55°03'01.89"W	*Pereira 1015*, MG*
Isoëtes cangae	Amendoim Lake	06°24'03.73"S, 50°22'23.27"W	*Campos 36*, RFA
Isoëtes serracarajensis	N3 flooded area	06°02'44.90"S, 50°12'34.68"W	*Campos 33*, RFA
Isoëtes serracarajensis	N6 flooded area	06°07'33.97"S, 50°10'39.43"W	*Campos 34*, RFA
Isoëtes serracarajensis	ISV flooded area	06°24'31.39"S, 50°21'05.38"W	*Campos 35*, RFA
Isoëtes serracarajensis	S11 flooded area	06°22'33.90"S, 50°23'00.34"W	*Campos 41*, RFA

* MG = Goeldi Museum (Museu Paraense Emílio Goeldi); RFA = Herbarium of the Federal University of Rio de Janeiro.

and *I. serracarajensis*. The samples were fixed in Karnovsky solution (Karnovsky 1965), dehydrated in an ethanol series, and embedded in plastic resin (Leica Historesin). Longitudinal and transverse section (8 µm thick) were obtained with a manual rotary microtome (Spencer 820, American Optical Corporation, Buffalo, NY, USA). The sections were stained with 0.05% toluidine blue O. in 0.1 M phosphate buffer, pH 6.8 (O'Brien *et al*. 1965) for 30 minutes and mounted on slides with Permount synthetic resin (Fisher Scientific®). Leaf epidermal peels of all three species were also prepared for stomatal observation. Transverse sections of fresh leaves were obtained by hand using a razor blade for histochemical tests. A 0.02% aqueous solution of ruthenium red was used to detect pectins (Johansen 1940). Observations and photographic records were performed and collected with an optical microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus DP71).

The following parameters were evaluated in the leaf transverse section of embedded samples: leaf width, leaf depth, leaf width/depth ratio, leaf area, lacuna sum area, leaf tissue area, number of peripheral fiber strands, interstellar canal width, interstellar canal depth, interstellar canal width/depth ratio, number of extrastellar canals, epidermal cell width, epidermal cell height, and epidermal cell width/height ratio. The number of cell layers in the trabeculae was quantified from longitudinal leaf sections. The root diameter, lacuna diameter, root/lacuna diameter ratio, root lacuna area, cortex thickness, number of cortex layers, vascular bundle diameter, and endodermis height were measured in the transverse section.

The leaf area, leaf lacuna sum area, and root lacuna area were calculated using ImageJ image analysis software (Schneider *et al*. 2012). The other micro-morphometric measurements were made using Image-Pro Plus v.4.5 software (Media Cybernetics, Silver Spring, USA).

Scanning electron microscopy

Leaf samples fixed in Karnovsky solution (Karnovsky 1965) were dehydrated in an ethylic series and dried to the critical point with CO_2 (equipment model CPD 030, Bal-Tec, Balzers, Liechtenstein). Then, the samples were attached to stubs with double-sided tape and coated with gold (equipment Sputter Coater, Quorum Technologies Ltd., Q150RS, Ashford, Kent, England.). Images were observed and captured on a scanning electron microscope (Zeiss, LEO 1430 VP, Cambridge, England).

Statistical analyses

The Shapiro-Wilk test was used to check for normality. To verify possible differences in micro-morphometric parameters among species/populations, we carried out a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for parametric data and the Kruskal-Wallis test followed by Dunn's multiple comparisons test for nonparametric data ($p < 0.05$). Statistical analyses were performed by GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA).

Results

Leaf and root characterizations are presented as separate topics. Micro-morphometric parameters were compared statistically, and the results are shown in Table 2. Additionally, the minimum and maximum of these quantitative parameters for the three species can be found in Table S1 (available on supplementary material <https://doi.org/10.6084/m9.figshare.24430951.v1>).

Table 2 – Leaf parameters analyzed in the *Isoëtes* species from Pará state, Brazil.

Characters	Species / Population					
Leaf parameters	*I. amazonica*	*I. cangae*	*I. serracarajensis* - N3	*I. serracarajensis* - N6	*I. serracarajensis* - S11	*I. serracarajensis* - ISV
Leaf width (mm)	1.03 ± 0.12 cd	0.96 ± 0.02 d	1.28 ± 0.18 b	1.25 ± 0.09 bc	0.96 ± 0.01 d	2.14 ± 0.06 a
Leaf depth (mm)	0.72 ± 0.04 c	0.91 ± 0.08 bc	0.98 ± 0.12 b	1.03 ± 0.09 b	0.75 ± 0.05 c	1.70 ± 0.08 a
Leaf area (mm^2)	6.14 ± 1.03 cd	8.38 ± 2.18 bd	9.55 ± 2.09 bc	10.2 ± 1.58 b	5.01 ± 0.36 d	27.2 ± 1.27 a
Lacunae area (mm^2)	2.54 ± 0.80 cd	3.30 ± 0.77 bd	4.00 ± 0.84 bc	4.99 ± 1.04 b	1.54 ± 0.37 d	13.1 ± 1.24 a
Lacunae / leaf area (%)	40.7 ± 9.33 ab	39.6 ± 2.36 ab	42.0 ± 4.46 ab	48.9 ± 4.33 a	30.4 ± 6.04 b	48.4 ± 3.62 a
Number of peripheral fiber strands	2 ~ 6	0	0	0	0	0
Number of interstellar canals	1 ~ 5	3 ~ 4	3 ~ 4	1 ~ 4	1 ~ 4	1 ~ 4
Interstellar canal width (µm)	34.8 ± 6.66 b	25.7 ± 4.37 b	37.8 ± 8.40 b	43.1 ± 13.4 b	28.1 ± 8.73 b	66.3 ± 2.52 a
Interstellar canal depth (µm)	30.6 ± 2.67 ac	37.0 ± 8.00 ab	28.5 ± 3.50 bc	31.7 ± 8.27 ac	24.7 ± 4.47 c	42.8 ± 2.37 a
Interstellar canal ratio	1.14 ± 0.18 ab	0.70 ± 0.05 b	1.35 ± 0.40 a	1.37 ± 0.12 a	1.16 ± 0.19 ab	1.56 ± 0.06 a
Number of accessory canals	4 ~ 6	5	5 ~ 6	6 ~ 7	2 ~ 6	5 ~ 8
Epidermal cells width (um)	29.3 ± 3.66 ab	17.8 ± 2.70 d	26.3 ± 3.15 ac	23.7 ± 3.12 c	25.5 ± 3.18 bc	30.0 ± 4.67 a
Epidermal cells height (um)	24.3 ± 2.92 a	16.7 ± 2.30 b	20.3 ± 3.57 ab	19.9 ± 5.16 b	20.3 ± 3.44 ab	20.1 ± 3.04 b
Diafragma cell layers	1 ~ 3	2 ~ 4	1 ~ 3	2 ~ 3	2 ~ 3	2 ~ 3

Legend: Values refer to Mean (± Standard Deviation) (n = 4). Different letters indicate statistical difference between species and populations for each parameter ($p < 0.05$).

Leaves

Leaf shape in transverse section varied among the Amazonian *Isoëtes* species and populations from Pará state (Fig. 2). *Isoëtes amazonica* had trapezoidal leaves (Fig. 2e), whereas *I. cangae* and *I. serracarajensis* from N3, N6, and ISV had semiterete to quadrangular leaves (Fig. 2a-c,f) and *I. serracarajensis* from S11 had semiterete to rounded leaves (Fig. 2d). Four aerenchyma lacunae crossed the leaves of all species.

Plants of *Isoëtes serracarajensis* from ISV had larger leaf width, leaf depth, and leaf and lacunae areas than plants of the other populations and species (Tab. 2). *Isoëtes amazonica*, *I. cangae*, and the population of *I. serracarajensis* from S11 had the lowest values for leaf width, leaf depth, and leaf and lacunae areas (Tab. 2). The lacunae/leaf area ratio was the lowest for *I. serracarajensis* from S11, and the other species and populations did not differ (Tab. 2).

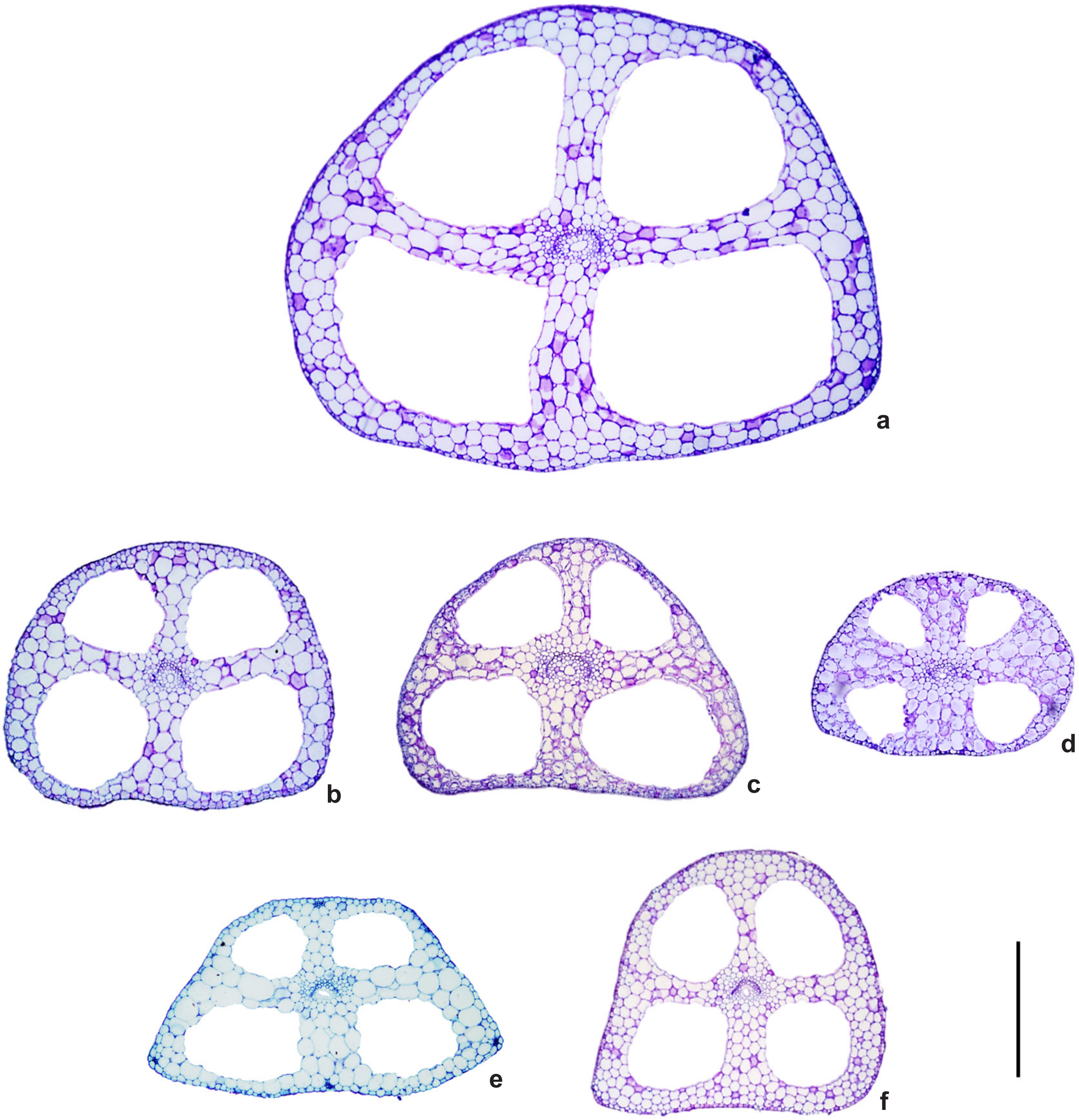

Figure 2 – a-f. Transverse sections of leaves of (a-d) *Isoëtes serracarajensis* from (a) ISV, (b) N3, (c) N6, and (d) S11 flooded areas, (e) *Isoëtes amazonica* and (f) *Isoëtes cangae*. Scale bar = 500 µm.

In all species, the four lacunae surrounded the leaf stele, which consisted of a single centralized vascular bundle (Fig. 3a-c). Within the stele of all analyzed species, one large centralized interstellar canal was present, and two to four smaller canals could also be observed in some samples (Tab. 2; Fig. 3a-c). Plants of *I. serracarajensis* from ISV had larger interstellar canal widths and depths than plants of the other populations and species, and *I. cangae* had the lowest interstellar canal width/depth ratio (Tab. 2). Vascular tissue organization in the leaf stele occurred as follows: the xylem formed an arc around the interstellar canals, and the phloem was externally distributed to the xylem arc extremities, creating two poles (Fig. 3a-c). The arc extremities were directed toward the adaxial face.

Externally to the leaf stele, a variable number of canals similar to but smaller than the interstellar canal was observed in all species and populations, hereafter called accessory canals (Fig. 3a-c). The smallest number of accessory canals - 2 - was observed in the leaves of *I. serracarajensis* from S11, and the largest - 8 - was observed in the population of this species from ISV (Tab. 2).

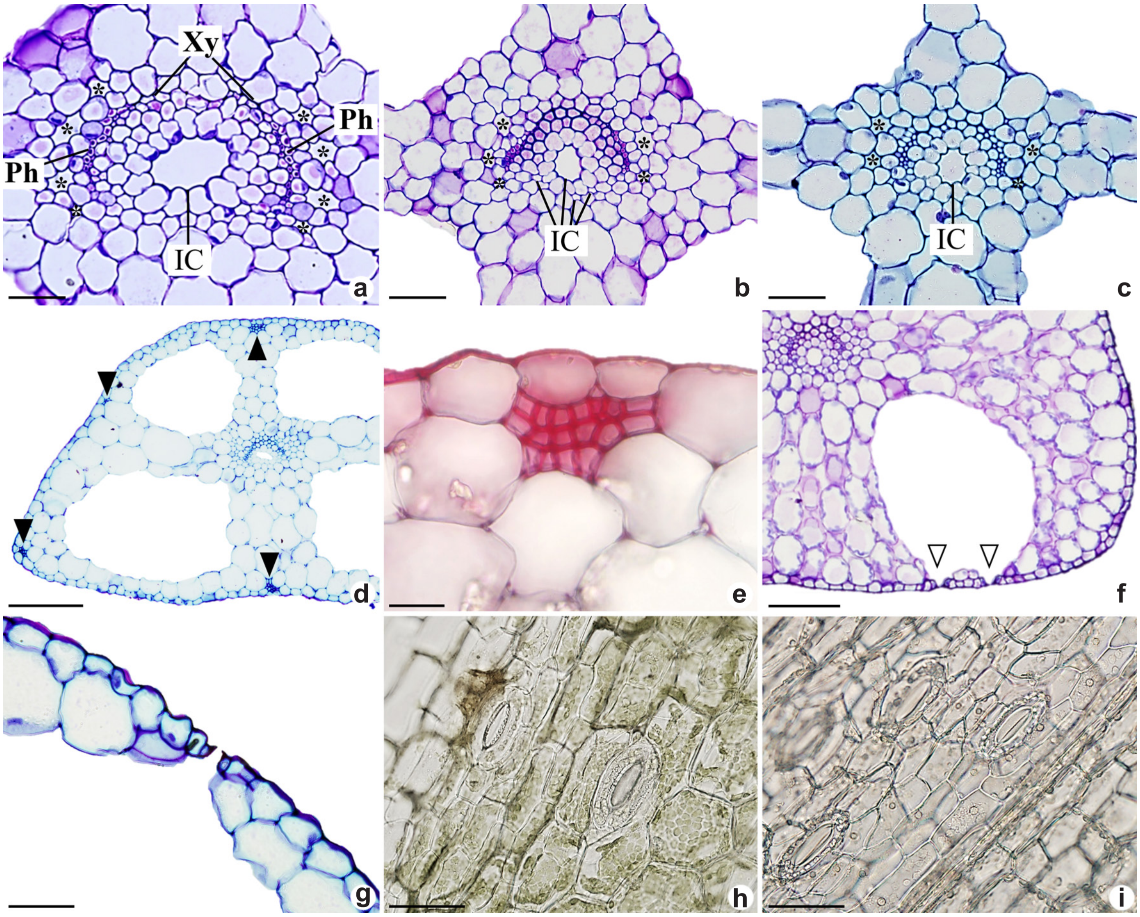

Figure 3 – a-i. Transverse sections (a-g) and epidermal peels (h-i) of leaves of the *Isoëtes* species from Pará, Brazil – a-c. leaf stele of (a) *Isoëtes serracarajensis* from the ISV flooded area; b. *Isoëtes cangae*; c. *Isoëtes amazonica*; d. peripheral fiber strands (black arrows) on an *Isoëtes amazonica* leaf; e. details of a peripheral fiber strand with thickened wall cells stained with ruthenium red on the abaxial face of an *Isoëtes amazonica* leaf; f. anomocytic stomata (white arrows) connected to adaxial lacunae on the leaf of *Isoëtes serracarajensis* from the S11 flooded area; g. details of stomata connected to abaxial lacunae on *Isoëtes amazonica* leaves; h-i. epidermal peels showing anomocytic stomata on leaves of (h) *Isoëtes serracarajensis* from the S11 flooded area; i. *Isoëtes amazonica*. Scale bars: a-c, e = 10 µm; d = 200 µm; f = 10 µm; g-i = 5 µm. Legend: IC = interstellar canal; Ph = phloem; Xy = xylem; asterisk (*) = accessory canal.

In *Isoëtes cangae* and *I. serracarajensis*, the mesophyll was composed of chlorenchyma, and peripheral fiber strands were absent. For *I. amazonica*, in addition to abundant chlorophyll parenchyma, a variable number of peripheral fiber strands was also observed below the leaf epidermis, ranging from two to six groups (Tab. 2; Fig. 3d-e). When found in number of six, the fiber strands were distributed with two centralized on the leaf and four at the leaf vertices, half on the adaxial face and the other half on the abaxial face of leaves. The positive reaction of fibers to the histochemical test with ruthenium red, used for pectin detection, indicated that they were collenchyma cells (Fig. 3e).

The epidermis of all species was uniseriate. *Isoëtes amazonica* and *I. serracarajensis* from N3 and ISV had the widest and tallest epidermal cells (Tab. 2). *Isoëtes cangae* had the narrowest epidermal cells, whereas the other species and populations had similar-sized cells (Tab. 2). Anomocytic stomata, connected to the air chambers of abaxial and adaxial leaf faces, were observed on the median and apical regions of the leaves of *I. amazonica* and *I. serracarajensis* (Fig. 3f-i). However, leaves of both species grown under full submergence did not present stomata. Stomata were absent in *I. cangae*.

The lacunae in each species and population were crossed by trabeculae along the leaf length (Fig. 4a-b). Trabeculae were formed by a variable number of braciform parenchyma cell layers; the smallest number of layers - 1 - was observed in N3 samples of *I. amazonica* and *I. serracarajensis*, and the largest number in *I. cangae* - 4 (Tab. 2; Fig. 4c). The braciform parenchyma cells were chlorophyllous and presented five to eight arms (Fig. 4d-g). Triangular pores were formed between braciform cell junctions, and intercellular pectic projections of filaments and connection types could be observed in all species (Fig. 4d-i). Pectic projections were more dense on the trabeculae of *I. cangae* (Fig. 4g).

The epidermis presented different ornamentation patterns for each species/population. In *I. cangae*, a smooth cuticle was observed. The contact walls of the epidermal cells were thickened, forming elevations that surrounded every cell (Fig. 5a). In *I. amazonica* and the populations of *I. serracarajensis* from N6, S11, and ISV, the cuticle showed a verrucate pattern spread above the leaf surface (Fig. 5b-d). In the *I. serracarajensis* population from N3, cuticle-formed tubules and verrucae were also observed in the junctions between epidermal cells (Fig. 5e-f).

Roots

Mature roots of *Isoëtes amazonica*, *I. cangae*, and *I. serracarajensis* assumed a round-to-elliptical shape in transverse section (Fig. 6a-f). Root diameter was larger for *I. cangae* and *I. serracarajensis* from ISV than for the other species and populations (Tab. 3). No differences in tissue distribution were observed among the species (Fig. 6a-f).

The roots had a large aerenchyma cavity, which occupied a significant amount of root area (Fig. 6). *Isoëtes cangae* and *I. serracarajensis* from ISV had a largest root diameter than *I. amazonica* and the other populations of *I. serracarajensis*. Additionally, *I. cangae* and *I. serracarajensis* from ISV had larger lacuna diameters and areas than *I. serracarajensis* from S11. *Isoëtes serracarajensis* from N6 had a higher lacuna/root diameter percentage than *I. amazonica* and *I. serracarajensis* from S11 and ISV (Tab. 3; Fig. 6a-c). The root cortex surrounded the aerenchyma lacuna and comprised parenchymatic cells (Fig. 6). The most peripheral layers of the cortex presented a differentiated cell wall coloration compared to that of the most internal layers. The species presented an overlap in the number of layers of parenchymal cells forming their cortex, with the largest number of layers - 5 - observed in plants from ISV (Tab. 3). The root cortex was thicker in *I. serracarajensis* from ISV than in plants from N6 (Tab. 3).

The vascular bundle was reduced and assumed an eccentric position in the root (Fig. 6). It was surrounded by the endodermis and had a collateral structure, with a single bundle of xylem arranged closer to the cortex and an arc of phloem with its convex side turned toward the lacuna (Fig. 6). The diameter of the root vascular bundle and endodermis thickness did not show any significant differences among the *Isoëtes* species and populations (Tab. 3).

Discussion

This study evaluated qualitative and quantitative leaf and root morphoanatomical parameters for the three *Isoëtes* species endemic to the Amazon Basin, Pará. Statistical comparisons were performed to confirm differences and similarities among the species. Anatomical characteristics were discussed based on ecological and genetic information and accounting for reports in the literature for other *Isoëtes* species.

The trapezoidal leaf shape in transverse section and the peripheral collenchyma fibers are the main anatomical leaf traits that differentiate *Isoëtes amazonica* from *I. cangae* and *I. serracarajensis*. These collenchyma fibers have been associated with ecological conditions where isoetids occur more frequently and are more pronounced in terrestrial and amphibious taxa. When present, the fibers act as a mechanically strengthening tissue supporting the leaves exposed to air over the water (drier atmospheric conditions) (West & Takeda 1915; Pfeiffer 1922; Kott & Britton 1985). *Isoëtes amazonica* is an amphibious species whose habitat may entirely dry out during the driest and hottest periods in the Amazon basin (Marengo & Espinoza 2016; Pereira *et al*. 2019), so the presence of fibers allows its emerged leaves to remain erect in the beginning of dry periods. Other South American amphibious species, such as *Isoëtes pedersenii* H. P. Fuchs *ex* E. I. Meza & Macluf and *Isoëtes luetzelburgii* U. Weber, also have peripheral fiber strands (Macluf *et al*. 2010; Pereira *et al*. 2018), and the latter has been identified as the sister clade of *I. amazonica*, along with other *Isoëtes* species (Pereira *et al*. 2021).

Isoëtes serracarajensis is also an amphibious species, but peripheral fiber strands were not observed in any of the studied populations. *Isoëtes*

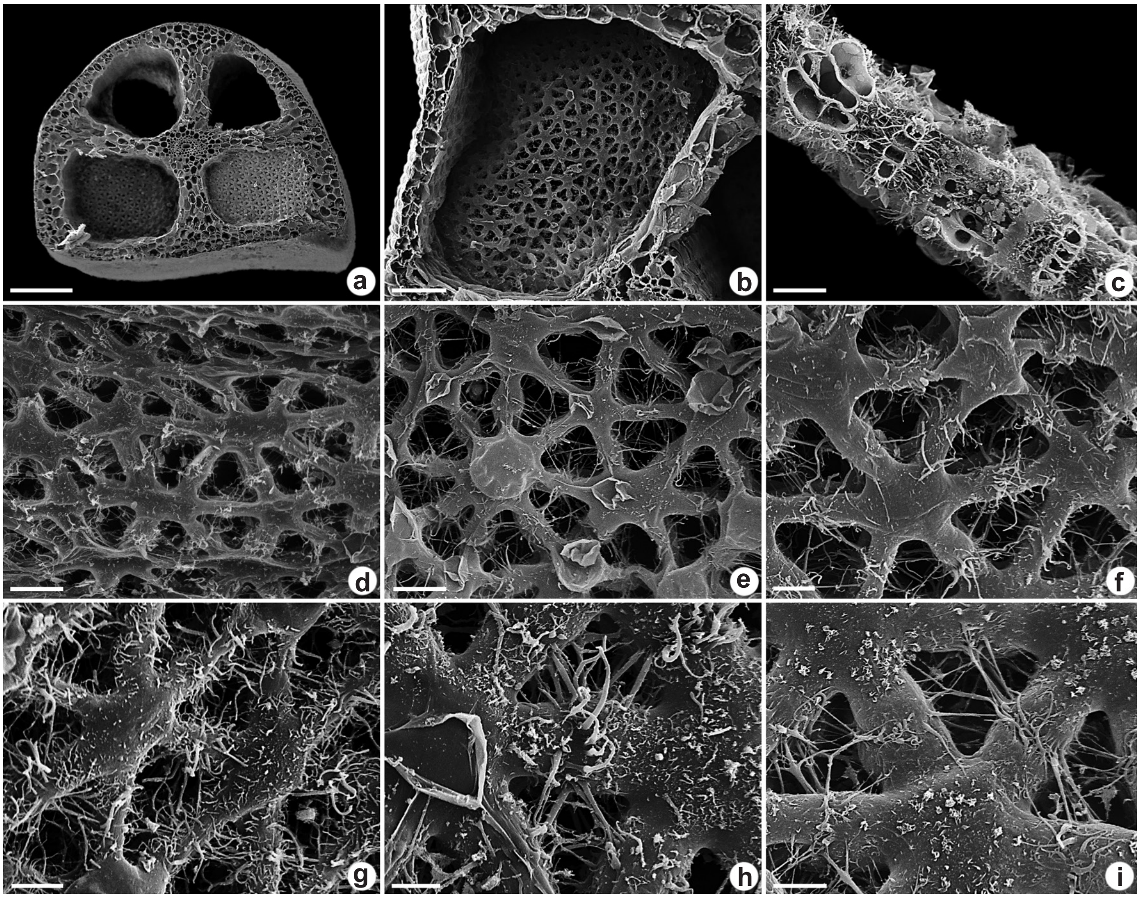

Figure 4 – a-i. Scanning electron microscopy transverse (a-b; d-i) and longitudinal sections (c) of leaves of *Isoëtes* species from Pará, Brazil – a. *I. serracarajensis* from ISV with two trabeculae present on the adaxial lacunae; b. details of the lacunae and trabeculae of *I. serracarajensis* from N6; c. leaf trabeculae of *I. cangae* showing 3–4 layers of braciform parenchyma cells; d-f. braciform cells forming triangular pores on the leaf trabeculae of (d) *Isoëtes amazonica*, (e) *Isoëtes serracarajensis* from N6 and (f) *Isoëtes serracarajensis* from S11; g. intercellular pectic projections densely present on *Isoëtes cangae* trabeculae cells; h-i. pectic projections of filaments (h) and connection (i) types on trabeculae of *Isoëtes serracarajensis* from ISV. Scale bars: a = 400 µm; b = 100 µm; c-e = 20 µm; f-i = 10 µm.

serracarajensis inhabits temporarily flooded areas that completely dry out for more extended periods than *I. amazonica*'s inundated shores (up to seven months, from April to October), such that the leaves of plants die while their corms remain protected and alive in the soil until the upcoming rainy season (Caldeira *et al*. 2021). Therefore, *I. serracarajensis* does not remain terrestrial for prolonged periods. New leaves are produced when their habitats are flooded again, where fiber strands are not usually found in other taxa. In addition, the absence of peripheral fibers may also be related to the aquatic species *I. cangae*, which also lacks this feature, being the potential maternal progenitor of *I. serracarajensis* (Pereira *et al*. 2021).

Isoëtes serracarajensis showed intraspecific variation in leaf shape, and plants from ISV had the highest values regarding the leaf and some of the root micro-morphometric parameters, especially when compared to the values of the S11 population. Intriguingly, both the ISV and S11 populations occur in the South Range of Serra dos Carajás, being geographically closer than the other populations (N3 and N6) from the North Range. Santos *et al*. (2020) analyzed the genetic diversity and population structure of the four populations of *I. serracarajensis*. They verified that plants from the ISV population had the highest percentage of polymorphic loci and the highest expected heterozygosity value, whereas plants from S11 had the lowest. Pereira *et al*. (2021) verified that *I. serracarajensis* is an allotetraploid species, which may first explain the genetic and anatomical diversity within this species, despite the proximity of its populations. Although all studied populations of *I. serracarajensis* inhabit the hydromorphic formations of Serra dos Carajás, sharing similar characteristics of geology and seasonality (Mota *et al*. 2015), intrinsic characteristics of each population may ultimately influence the anatomical diversity within this species. The depth of the water column and the area occupied by each water body directly determine the period in which water will remain in their habitats and prevent early plant exposure to high temperatures and evaporative demands, winds, and intense radiation. The S11 flooded area was the smallest and had the shallowest waters among the areas occupied by the *I. serracarajensis* populations. This may explain the observation of the smallest and least developed leaves on plants from this population.

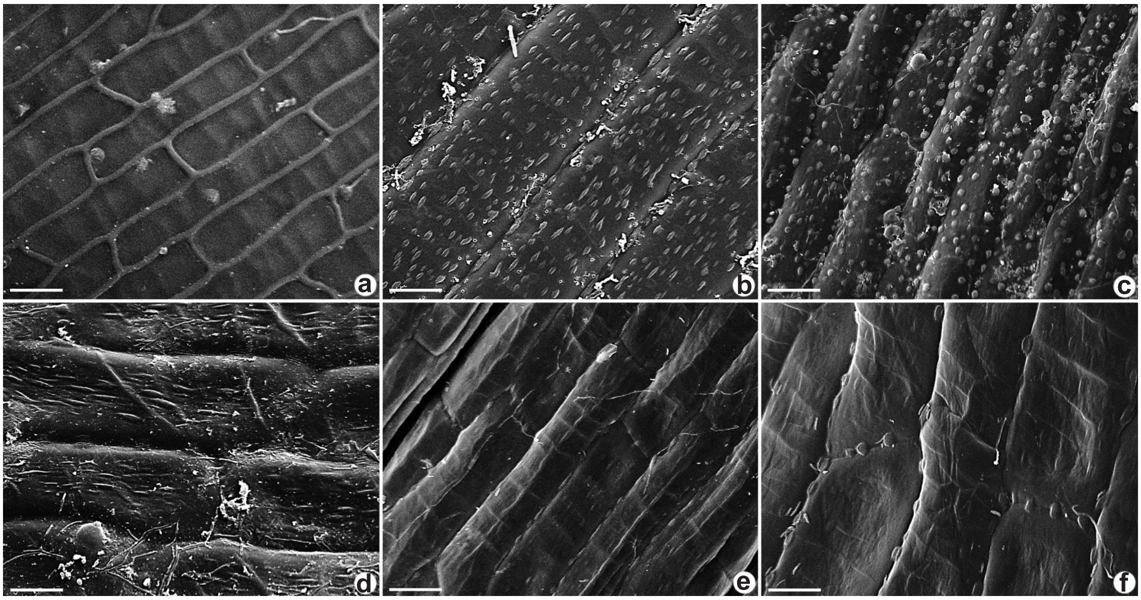

Figure 5 – a-f. Scanning electron microscopy of the leaf surface of *Isoëtes* species from Pará, Brazil – a. cuticle elevations surrounding *Isoëtes cangae* epidermal cells; b-d. verrucate cuticle pattern of (b) *Isoëtes amazonica* and *Isoëtes serracarajensis* from (c) S11 and (d) ISV; e-f. cuticle of *Isoëtes serracarajensis* from N3 forming tubules (e) and presenting verrucae in the junctions between epidermal cells (f). Scale bars: a, c = 20 µm; b, d-f = 10 µm.

On the other hand, plants from N3, N6, and ISV may have experienced higher water levels and presented more developed leaves, with larger leaf and lacunae areas.

The epidermal cell dimensions were the main leaf morphometric parameters used to differentiate *I. cangae* from *I. amazonica*. *Isoëtes cangae* had smaller epidermal cells than all *I. serracarajensis* populations, whereas *I. amazonica* had the largest epidermal cells. This finding may be linked to the carbon assimilation strategy required in their respective environments. In submerged plants, gas exchange occurs directly through the leaf epidermis since aquatic species usually lack stomata (Mommer *et al.* 2005; Klimenko 2012). Therefore, reducing cell size is essential for decreasing diffusion resistance in subaquatic leaves (Goliber & Feldman 1990; Mommer *et al.* 2005; Han *et al.* 2021). Another explanation may be related to their ploidy level. *Isoëtes cangae* is diploid, whereas *I. serracarajensis* and *I. amazonica* are tetraploids. Chromosome number may impact the size of the epidermal cells as it affects the size of spores (*e.g.*, Pereira *et al.* 2015; Barrington *et al.* 1986).

Cuticular ornamentations were present on the amphibious species *I. amazonica* and *I. serracarajensis*, whereas the aquatic species *I. cangae* has a smooth cuticle that favors gas diffusion. These findings are in accordance with those of Troia *et al.* (1999) and Prada & Rolleri (2003), who reported that cuticular papillae occur only in terrestrial species. In addition, the marginal thickening of the epidermal cell walls, observed only for *I. cangae* leaves, indicates a possible relation to its aquatic habitat. Additionally, Prada & Rolleri (2003) suggested that epidermal cellular groups with thickened walls are characteristics of aquatic taxa that allow mechanical sustentation, as these species usually lack collenchyma tissues.

Both *Isoëtes amazonica* and *I. serracarajensis* presented stomata, whereas *I. cangae* did not.

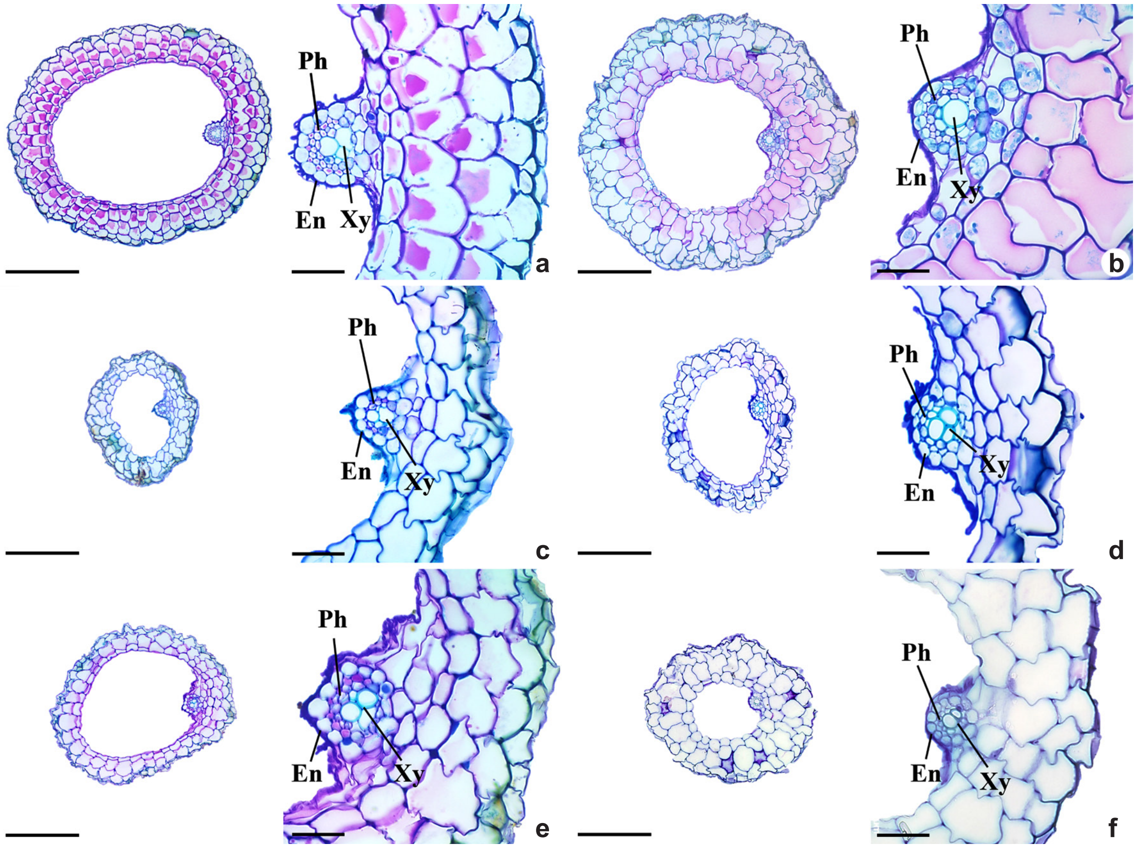

Figure 6 – a-f. Transverse sections of roots (on the left) and detailed imagens of the root vascular bundle (on the righ) of *Isoëtes cangae* (a), *Isoëtes serracarajensis* from (b) ISV, (c) S11, (d) N3 and (e) N6 flooded areas, and *Isoëtes amazonica* (f). Scale bars = 250 µm (left); 10 µm (right). Legend: En = endodermis; Ph = phloem; Xy = xylem.

Table 3 – Root parameters analyzed in the *Isoëtes* species from Pará state, Brazil.

Characters	**Species / Population**					
Root parameters	*I. amazonica*	*I. cangae*	*I. serracarajensis* - N3	*I. serracarajensis* - N6	*I. serracarajensis* - S11	*I. serracarajensis* - ISV
Root diameter (mm)	0.57 ± 0.12 b	0.79 ± 0.10 a	0.52 ± 0.08 b	0.56 ± 0.10 b	0.46 ± 0.10 b	0.87 ± 0.05 a
Lacunae diameter (mm)	0.33 ± 0.10 ab	0.53 ± 0.08 a	0.34 ± 0.07 ab	0.41 ± 0.07 ab	0.27 ± 0.07 b	0.51 ± 0.09 a
Lacunae/Root diameter (%)	57.3 ± 8.77 b	67.3 ± 1.52 ab	65.8 ± 3.50 ab	73.4 ± 2.53 a	58.1 ± 3.36 b	59.0 ± 7.35 b
Lacunae area (mm^2)	0.08 ± 0.05 ab	0.21 ± 0.07 a	0.07 ± 0.02 ab	0.12 ± 0.05 ab	0.05 ± 0.02 b	0.18 ± 0.06 a
Cortex thickness (µm)	133 ± 34.8 ab	136 ± 10.8 ab	155 ± 126 ab	80.0 ± 19.2 b	97.9 ± 20.1 ab	204 ± 29.5 a
Cortex layers	3 ~ 4	4	3	3	3 ~ 4	4 ~ 5
Vascular bundle diameter (µm)	41.8 ± 12.8 a	46.7 ± 4.07 a	47.1 ± 8.31 a	44.7 ± 9.64 a	29.0 ± 6.50 a	44.0 ± 4.56 a
Endodermis thickness (µm)	12.8 ± 2.24 a	12.6 ± 1.27 a	13.5 ± 2.81 a	13.2 ± 3.87 a	11.6 ± 1.34 a	12.3 ± 0.76 a

Legend: Values refer to Mean (± Standard Deviation) ($n = 4$). Different letters indicate statistical difference between species and populations for each parameter ($p < 0.05$).

Isoëtes amazonica and *I. serracarajensis* are amphibious species, and their leaves develop under both submergence and emergence. For the North American *Isoëtes* species, Kott & Britton (1985) considered the presence of stomata as variable and dependent on habitat conditions and suggested that stomata are formed on developing leaves exposed to the air. Stomatal presence has also been noted to prevent catastrophic xylem embolism in air-exposed leaves by limiting the maximum evaporative flux (Tyree & Sperry 1998), allowing the continuity of the water column in the xylem conduits. In agreement, *Isoëtes* is the largest group of plants with aquatic CAM and the lycopsid photosynthetic pathway (LPP; see Green 2010), resembling the features of arborescent lycopsids of the Paleozoic (Keeley 1981; Keeley & Bowes 1982; Green 2010). This suggests that *I. amazonica* and *I. serracarajensis* are CAM-like species, so stomata play an essential role in storing CO_2 when leaves emerge (Green 2010). This strategy enables stomata to open at night and stay closed during the day, when the vapor pressure deficit is much higher, thereby avoiding unnecessary water loss (Keeley 1981).

The air lacunae occupied more than 30% of the leaf area and more than 55% of the root diameter. The air lacunae on the leaves and roots of the genus play an essential role in habitat adaptation for aquatic and terrestrial species. Phillips & DiMichele (1992) suggested that *Isoëtes*' aerenchyma may act as an internal system of gas exchange associated with photosynthesis capacity, essential for CO_2 uptake via stomata or diffusion through the epidermis. The physical connection between stomata and the air chambers, clearly observed in *I. amazonica* and *I. serracarajensis* leaves, confirms that lacunae replace the substomatal cavity, providing a CO_2 source for CAM photosynthesis. In plants growing submerged or in wetland environments, root aerenchyma is also important to complement CO_2 uptake from the rhizosphere (Winkel & Borum 2009) and to allow root aeration, which would not be possible without a downward oxygen transport system (Phillips & DiMichele 1992; Green 2010).

A variable number of interstellar and accessory canals, surrounded by a distinct endodermal layer, could be seen in leaf transverse section of all species. Until now, accessory canals outside the xylem/phloem arc had not been reported in the literature. These traits may show slight variations, and authors such as Budke *et al*. (2005) considered the number of interstellar canals to be stable on the leaves of *I. tennesseensis* Luebke & Budke, presenting rare variations from one to two canals. Additionally, Hall (1971) and Takamiya *et al*. (1997) found that the number of canals varied among *Isoëtes* species from Ghana and Japan, respectively, and the canals were noted as a good taxonomic character by the authors. Although the function of the interstellar canals has not been elucidated, Hall (1971) observed that these canals were filled with water and inferred that they functioned similarly to xylem vessels. Similarly, Romeo *et al*. (2000) inferred that these canals would be related to the canalization of rising water due to root pressure in submerged plants of *Isoëtes* and in the stomata transpiration flux mechanism in terrestrial or amphibious species.

Diaphragms formed by braciform cells with similar intercellular pectic projections were observed on the leaves of *I. amazonica*, *I. serracarajensis*, and *I. cangae*. Although the function of these differentiated structures has not yet been elucidated in *Isoëtes*, the diaphragms have been noted as necessary for protection against internal flooding when leaves are damaged, for air circulation, allowing air passage through pores, and for mechanical support in aquatic plants (Snow 1914; Armstrong *et al*. 1988; Soukup *et al.* 2000). Mechanical support may be essential for *I. cangae*, which grows submerged and under exposure to a high-water column pressure and does not have peripheral fibers. *Isoëtes cangae* was the only species to present up to four layers of braciform cells on the translacunar diaphragm. Additionally, the pectic projections were denser in this subaquatic species than in the other amphibious ones, guaranteeing additional stability for the diaphragms. Different authors have suggested that the intercellular pectic projections, which present both hydrophilic and lipophilic properties, are essential for cell wall hydration, storage, cellular adhesion, plant defense, apoplastic transport, and the maintenance of structural tissue integrity (Heide-Jørgensen 1978; Davies & Lewis 1981; Morris *et al*. 1982; Potgieter & Van Wyk 1992; Machado & Sajo 1996).

Isoëtes serracarajensis shares adaptive traits with both *I. cangae* and *I. amazonica*, the appearance of which depends on environmental conditions. The species has a leaf shape similar to that of the aquatic species *I. cangae* and lacks peripheral fiber strands normally present in terrestrial/amphibious taxa. On the other hand, similar to the amphibious species *I. amazonica*, *I. serracarajensis* presents stomata and cuticular ornamentations and has similar epidermal cell dimensions. Anatomical variations among *I. serracarajensis* populations may be explained by their tetraploidy and genetic diversity, which the intrinsic characteristics of their habitats may ultimately favor.

In our study, we verified that the Amazonian *Isoëtes* species present anatomical adaptations to the habitats in which they live. South America is home to a great diversity of plants from this genus, but to date, most species have been neglected and poorly studied regarding their anatomy. We suggest that further studies evaluating the anatomical traits of Neotropical *Isoëtes* species could clarify their ecology, genetics, and phylogeny. Understanding the relationships between plant anatomy and habitat is essential for developing both *in situ* and *ex situ* conservation strategies for threatened and data-deficient species in a scenario of human impacts on their habitats.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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