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Distinct Reproductive Strategy of Two Endemic Amazonian Quillworts

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Abstract: We examined the reproductive strategy of two Amazonian quillworts (*Isoëtes cangae* and *Isoëtes serracarajensis*), endemic and threatened species of *canga* ecosystems. Sexual propagation was examined by in vitro fertilization assays, while asexual propagation was examined by tiller emission. *Isoëtes cangae* is an outcrossing species that reproduces exclusively by spore germination and is able to propagate by self- and cross-fertilization. *Isoëtes serracarajensis* reproduces asexually by emitting tillers from the plant corm, despite producing male and female sporangia. These distinct reproductive strategies in the different species may be linked to their contrasting habitats. *Isoëtes cangae* inhabit a permanent oligotrophic lake with mild environmental changes, while *I. serracarajensis* are found in temporary ponds facing severe seasonal drought, where asexual propagation may represent an adaptive advantage to the short growth period during access to water. We also observed different relationships between plant growth and reproductive traits between the species, despite their common production of sporophytes with high survival rates. Together, these results are of paramount importance for establishing conservation plans for both species considering the advantages of sexual propagation to maintain the genetic diversity of *I. cangae* and the diligent management required to do the same with asexually propagated *I. serracarajensis*.

Keywords: *Isoëtes*; propagation; conservation; *canga*; Carajás

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1. Introduction

The *Isoëtes* L. genus constitutes heterosporous lycophytes widely distributed globally, occurring essentially in aquatic environments and in seasonally flooded areas [1,2]. This group was dominant in the Carboniferous period, forming some of the most extensive plant fossil deposits of any geological period [3]. South America is considered a significant center of taxonomic diversity, and Brazil is a prominent region, with at least 26 *Isoëtes* species [4,5].

Isoëtes cangae and *Isoëtes serracarajensis* were recently described as endemic species of *canga*, the ferruginous mountain outcrops of Serra dos Carajás, Brazilian eastern Amazon [1,6]. Such mountain outcrops harbor several phytophysiognomies (grasslands, scrublands, wetlands, and forest formations [7]), facing severe environmental conditions (high temperature, UV radiation, high evapotranspiration, and poorly developed soils rich in

metals [8]), harboring endemics and/or rare species [9]. *Isoëtes cangae* is an aquatic species found submerged only in a permanent lake, while *I. serracarajensis* is more widely distributed and found partially or totally submerged in temporary ponds or humid areas on four plateaus of Serra dos Carajás (Serra Sul, Serra Norte, Serra da Bocaina and Serra do Tarzan) [1]. Because of its restricted distribution and habitat quality deterioration triggered by alterations in the hydroclimatological cycle in the region (prominent forest conversion into pasturelands and mining activities [1,10]), *I. cangae* was recently classified as a critically endangered species (CR) according to the International Union for Conservation of Nature (IUCN) criteria [11], while *I. serracarajensis* is still not listed.

The development of propagation studies and the determination of their genetic structure and diversity have been the first steps toward the conservation of rare and endemic *Isoëtes* species [12–16]. The in vitro generation of new *Isoëtes* plants can occur by sexual reproduction [14,15,17] and, more rarely, by asexual or vegetative reproduction, usually observed by the division of the corm [18,19]. Sporophyte regeneration by sexual reproduction has been more frequently observed and is widely used for in vitro propagation [13–15,17,20]. Meanwhile, asexual propagation have also been observed and described as fundamental in the reproduction of *Isoëtes andicola* [18] and *Isoëtes savatieri* [4].

The understanding of propagation, growth, and development is of paramount importance to the establishment of conservation plans for the rare and endemic species *I. cangae* and *I. serracarajensis*. While Caldeira et al. (2019) have reported sexual propagation for *I. cangae* by self-fecundation, studies on cross-fertilization, apomixis, and vegetative propagation are still missing. Furthermore, there are no results available regarding *I. serracarajensis* reproduction. Here, we investigated the reproductive strategy of both species and outlined (i) the impact of seasonality on spore germination; (ii) the influence of apomixis, self-fertilization, and cross-fertilization on sporophyte regeneration and growth; (iii) vegetative propagation; and (iv) the pattern of ex situ growth and sporangia and tiller production.

2. Materials and Methods

2.1. Plant Material

Plants of *I. cangae* were collected from the four portions (north, south, east, and west) of the Amendoim Lake (Serra Sul) in Serra dos Carajás, Brazil (Figure 1) during the dry (June to October) and rainy seasons (November to May). Permission to collect the specimens was granted by the Chico Mendes Institute of Biodiversity of the Ministry of the Environment (ICMBio/MMA; numbers 641873 and 59724; SISGEN- ADC5012). *Isoëtes serracarajensis* was found and collected only during the rainy season in the temporary ponds of four different iron-rich mountain outcrops harboring the *canga* ecosystems of Carajás: Serra Norte, Serra Sul, Serra do Tarzan, and Serra da Bocaina (Figure 1). A total of 220 plants of *I. cangae* and 80 plants of *I. serracarajensis* were collected. In the laboratory, the plants were transferred to transparent plastic containers (volume of 2 dm^3) containing substrates from their respective lake (or pond) and covered with distilled water. Some of them were maintained in controlled environmental conditions (SCG 120, Weiss Technik, UK–12:12 h photoperiod, 80 µmol m^{-2} s^{-1} photosynthetic photon flux density and 28:22 °C day:night temperature regime), while others were maintained in greenhouse conditions.

2.2. Spore Germination from Sporangia Collected during the Dry and Rainy Seasons

To outline the influence of seasonality on megaspore germination and in vitro fertilization, 300 mature megaspores of *I. cangae* (30 plants) were used per collection period (dry or rainy), and 200 megaspores of *I. serracarajensis* (20 plants), collected only in the dry season. The number of megaspores used was lower in *I. serracarajensis* due to the smaller number of fertile individuals collected. Sporangia collection and sterilization were carried out as described by Caldeira et al. (2019). Briefly, after gently removing the intact sporangia from the leaf base, they were peeled away with forceps, treated with 70% ethanol for 1 min, rinsed with sterile distilled water, sterilized again for 3 min with a solution of NaClO (1%) containing Tween-20 (0.01%), and rinsed three times with sterile distilled water.

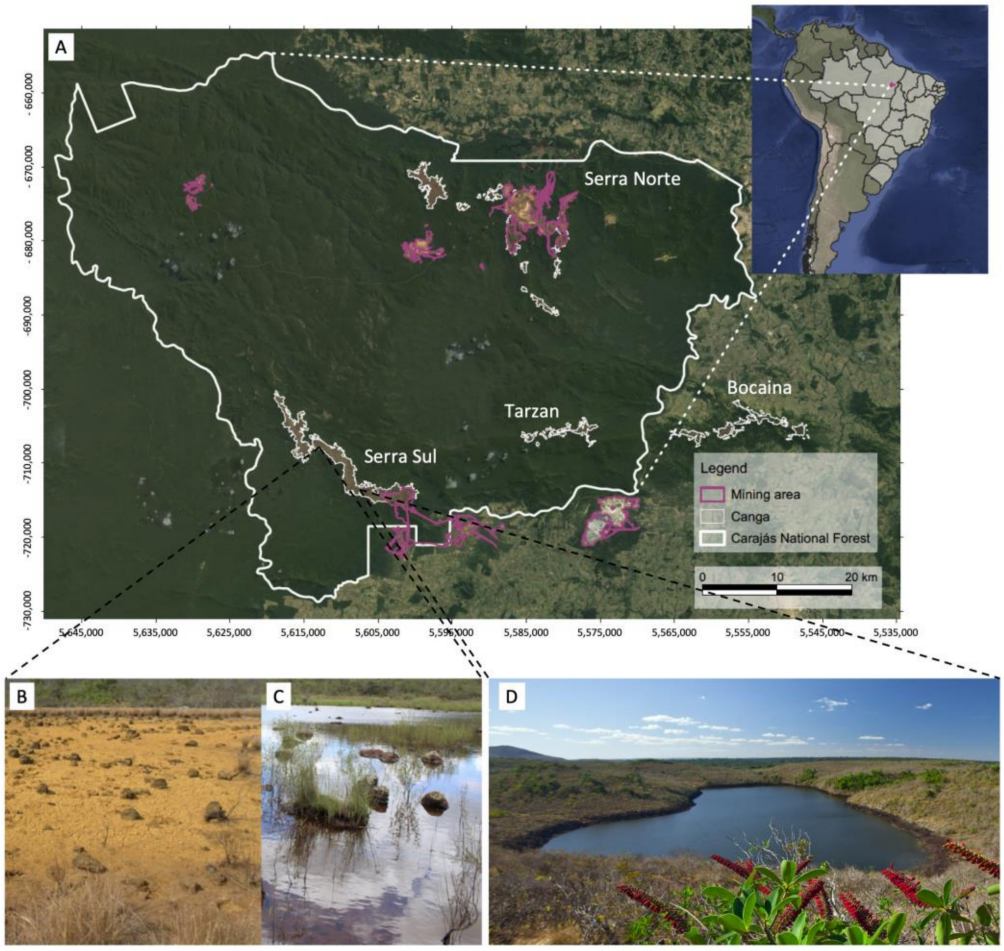

Figure 1. Map of the Carajás National Forest and the sites where *I. serracarajensis* and *I. cangae* were collected. (**A**) The mining areas and the four iron-rich mountain outcrops harboring the *canga* ecosystems of Carajás: Serra Norte, Serra Sul, Tarzan, and Bocaina. Details of the temporary ponds (sites with *I. serracarajensis*) during the (**B**) dry and (**C**) rainy seasons and (**D**) a permanent lake where *I. cangae* is submerged.

The megasporangia were then ruptured with forceps, and the spores were counted and incubated in 50 mL Falcon tubes filled with sterile distilled water. Then, microspores were also released from the sporangia and simultaneously added to the tubes. The tubes were kept in a growth chamber (SCG 120, Weiss Technik, Loughborough, UK) under the same environmental conditions described in Section 2.1. Megaspore germination was evaluated twice a week using a stereomicroscope, and fertilization was carried out with the germinated megaspores. The sporophyte appearance was checked and removed once every two days for 90 days. Young sporophytes were kept in distilled water until root emergence, when they were transferred to pots with substrate covered by a thin layer of sterilized quartzite sand to avoid the spread of debris [14].

2.3. Sexual Propagation Assays

To evaluate spore germination and in vitro sporophyte regeneration, we carried out two sets of experiments with both species. In all experiments, we evaluated: (i) self-fertilization (mixed megaspores and microspores of the same individual), (ii) cross-fertilization (mixed megaspores and microspores of different individuals), and (iii) apomixis (only megaspores isolated). Initially, we used mature spores collected from 13 adult plants of I. cangae (3.520 megaspores) and two plants of *I. serracarajensis* (448 megaspores). We carried out four tests for *I. cangae* and one for *I. serracarajensis*. The tests were conducted on different numbers of plants, following the availability of male

and female sporangia (Table S1). Sporangia collection and sterilization were carried out as described in Section 2.2, except for the apomixis treatment, which did not receive the addition of micropores. To check for megaspore viability in the apomixis treatment, the megaspores were observed under a stereomicroscope. Subsets of germinated megaspores (marked by a trilete aperture, exposure of the archegonia, and a rhizoid appearance) were isolated in sterile distilled water. Then, after 15 days of isolation, microspores were added to the tubes once no leaf emission was observed. The sporophyte appearance was checked and removed once every two days for 90 days.

In the second set of experiments, we examined the influence of microspore addition on megaspore germination. Treatments consisted of (i) megaspore germination in the presence of microspores, i.e., micropores added simultaneously to the mixture with megaspores, and (ii) megaspore germination in the absence of microspores, which were added after archegonia exposure and rhizoid emission. In this case, the germinated megaspores were periodically removed from the mixture and transferred to other tubes containing microspores. These experiments were carried out considering the effect of self- and cross-fertilization, totaling four treatments with 300 megaspores each for *I. cangae* e 100 megaspores for *I. serracarajensis* (10 megaspores collected per plant of each species). Because of the absence of megaspore germination for *I. serracarajensis*, we did not carry out the treatment involving microspore addition after germination. Apomixis tests were also performed with the same number of megaspores for each species. Sporangia collection and sterilization, spore incubation, and germination were carried out under the same environmental conditions as described previously. Likewise, the sporophyte appearance was checked and removed once every two days over 90 days, and the young sporophytes were transferred to pots with substrate and distilled water. After growing for 90 days, we counted the number of leaves and measured the plant height, root length, and fresh weight of the entire plant.

Nuclear DNA Extraction and PCR Amplification of ISSR Markers

The plants generated in the apomixis treatment were analyzed to compare them with the mother plants' total genomic DNA using ISSR molecular markers. For that, the total genomic DNA was isolated from 200 mg of leaves. The leaf tissues were macerated in liquid nitrogen, and total DNA extraction was performed following the modified CTAB protocol [21,22]. The DNA quality was evaluated with an agarose gel (1%) and its concentration was estimated using a Nanodrop®. The DNA samples (20 μg/μL) were used to amplify the DNA by PCR using genetic ISSR markers (Table S2). The proportion of the same or different bands within each family (the mother plant and its respective progeny) was observed, and the percentage of polymorphic bands between parent plants and progeny was calculated as described by Santos et al. (2020).

2.4. Isoëtes cangae Ex Situ Growth and Sporangia Production

Isoëtes cangae sporophyte growth and development were monitored in experiments carried out in a controlled environment (growth chamber) and under greenhouse conditions. Approximately two thousand sporophytes were continuously generated from the procedures described by Caldeira et al. (2019) and cultivated in growth shelters with the same environmental conditions described in Section 2.1. These young sporophytes (1 cm long) were cultivated in organic substrates (Jiffy-7®), lake sediments, and a mixture (1:1) of both substrates. In all cases, the substrates were covered with a layer of sterile quartzite sand, and distilled water was used to fill the containers. The sporophytes were monthly counted to estimate the survival rate and to check for new individuals. Some of these sporophytes (approximately 300 plants) were kept in these environmental conditions for a period longer than 24 months. Some of them were cultivated in groups of three plants in glass containers of 9 dm^3 (with 0.9 dm^3 of substrate), while others were isolated in plastic pots of 2 dm^3 (a single plant per pot, with 0.2 dm^3 of substrate). Fifty plants aged 12 months were randomly selected to determine the number of leaves and sporangia. The

leaves were manually detached from the plant base (corm) to check for the presence of sporangia.

In a second experiment, a group of 130 young (nonfertile) and 90 adult (fertile) plants collected in the field were transferred to glass containers (70 L) with 15 plants (young) or individually in plastic beakers of 4 L (adult). All sporangia from the adult plants were removed before planting. The plants were cultivated in a greenhouse for 12 months, using organic substrates (Jiffy-7®) and following the procedures suggested by Zandonadi et al. (2021). Sporangia production and sporophytes apparition was monitored during this period. The plant families were evaluated, and the progenies generated were analyzed and compared to the mother plants' total genomic DNA using ISSR markers.

2.5. Vegetative or Asexual Propagation Assays

Asexual propagation was evaluated in *I. cangae* during the cultivation of a large number of plants (Section 2.4) and in two series of experiments for *I. serracarajensis*. Initially, 25 young plants collected in the field were transferred to individual pots with a mixture of organic substrate (Jiffy-7®) and lake sediments covered by sterile quartzite sand. They were cultivated in growth shelters away from the *I. cangae* plants. After 12 months, we examined the appearance of new plants and counted the tillers attached to the corm of the mother plant. We counted the number of leaves and sporangia at their bases. Then, the tillers recovered were transferred to pots with substrate and cultivated under the same environmental conditions. Their survival was evaluated three months later.

The second experiment was carried out with 20 individuals of *I. serracarajensis* collected in the field and cultivated under greenhouse conditions in individual pots containing organic substrate (Jiffy-7®). After 12 months, we counted the tillers attached to the plant corm, collected the mother and daughter plants, and examined the families (mothers and daughters) by using ISSR markers.

2.6. Data Analysis

Data analyses were conducted in R version 3.5.2 [23]. We performed a Shapiro–Wilk normality test to define the subsequent parametric (Student's *t*-test) or non-parametric (Kruskal–Wallis rank test or Mann–Whitney U test) post-hoc tests. For spore germination in the three propagation methods (apomixis, self, and cross-fertilization) and to examine the influence of microspore addition on megaspore germination, we applied a post-hoc Dunn's test ($p < 0.05$). When applicable, the R function *lm()* was used to generate regression models. The figures were built using the R package ggplot2.

3. Results

3.1. Spore Germination and Sporophyte Growth from Sporangia Collected during the Dry and Rainy Seasons

Among the 300 megaspores of *I. cangae* collected during the rainy season, 175 germinated (58.3%). We found that megaspores germinated between 12 and 20 days by checking the megaspore opening for archegonia exposure (Figure 2A) and rhizoid emission (Figure 2B). Rhizoids were observed in large quantities both before and after fertilization and were no longer visible in the plants 30 days after the trilete aperture and archegonia exposition. After microspore addition to the tubes and fertilization, we found the generation of 170 individuals (97%). The first leaf and root emissions appeared on the twentieth day (±5 days) after fertilization (Figure 2C). We did not observe any germination of megaspores or sporophyte generation for *I. serracarajensis* in this experiment.

In plants collected during the dry season, we observed a higher proportion of immature megaspore spores (60 ± 5%) than mature spores (dark color) (Figure 2D). Megaspores from the dry season displayed germination, and sporophyte regeneration was delayed after two months (±5 days). The megaspore germination reached 46% (138 from the 300 megaspores), and the sporophyte regeneration was similar to that of plants collected in the rainy season (98%).

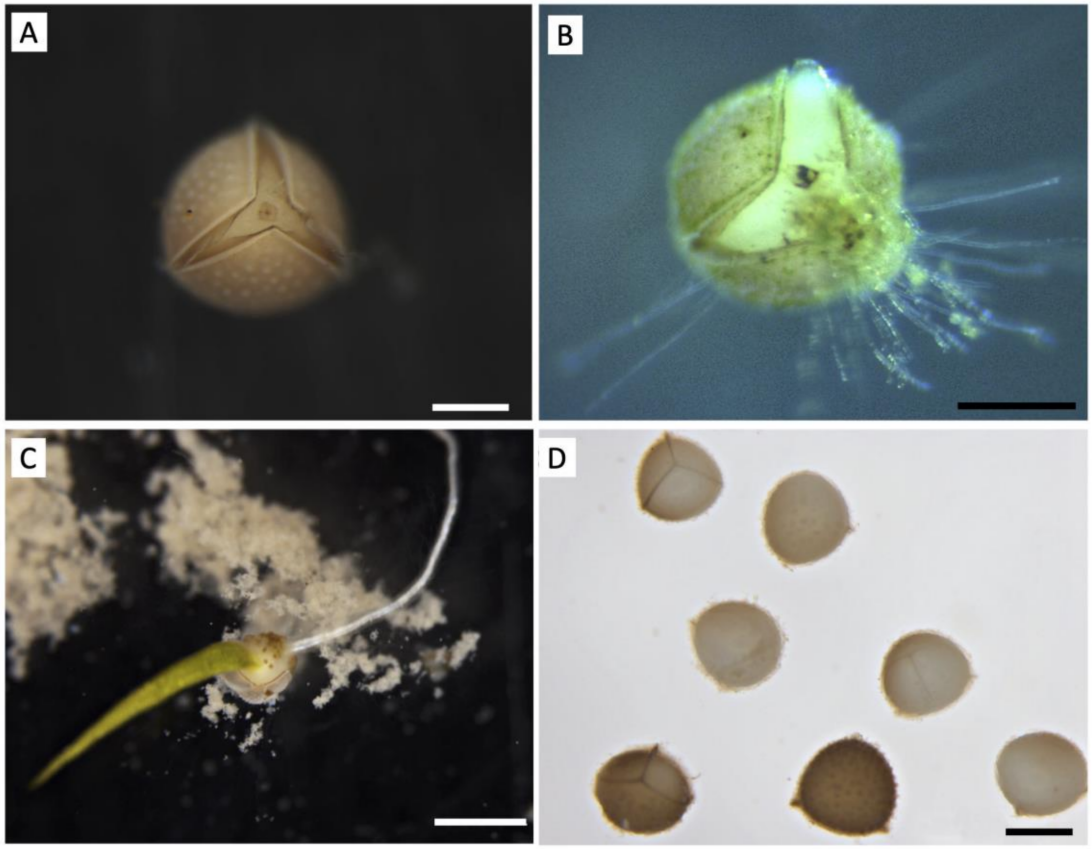

Figure 2. Megaspore germination and sporophyte formation in *Isoetes cangae*. (**A**) Megaspore opening with archegonia exposure; (**B**) megaspore germination with rhizoid emission; (**C**) first leaf and root emission after fertilization; (**D**) mature megaspores (dark) and immature megaspores (white). Scale bars: **A**,**B** = 200 µm; **C** = 1 mm; **D** = 500 µm.

3.2. Sexual Propagation and Progenies Growth

In the first series of experiments with *I. cangae* (four individual assays), we observed a large variation in the regeneration of sporophytes from self- and cross-fertilization treatments (from 0% to 90%), showing no significant differences between them (Figure 3A). Overall, in the apomixis treatment (absence of microspores), we did not observe the appearance of sporophytes, except for the 2% in one experiment, all of them from the same plant. In this treatment, the megaspore germination (as Figure 2B) attested for their viability. Moreover, subsets of such megaspores (kept isolated for 15 days) developed into sporophytes a few days after microspore addition (Figure S1). Nonetheless, we did not obtain sporophytes in all treatments carried out with *I. serracarajensis*.

In the second series of experiments, the percentage of progeny production was 93% and 80% for self-fertilization tests, with and without the addition of microspores before germination, respectively. For cross-fertilization, the percentages of sporophyte regeneration were 87% and 49% with and without the addition of microspores before germination, respectively (Figure 3B). *Isoëtes cangae* isolated megaspores spontaneously generated sporophytes (only two individuals in three trials of apomixis); however, the mother plants were different genotypes from the progenies, as confirmed by ISSR. The percentage of polymorphic bands between the parental plants and progenies was 65%. Sporophytes generated from cross-fertilization presented a greater height (leaf length), root length, and fresh weight than self-fertilization at 3 months of age (Figure 4A–C), despite a non-significant difference in the number of leaves (Figure 4D).

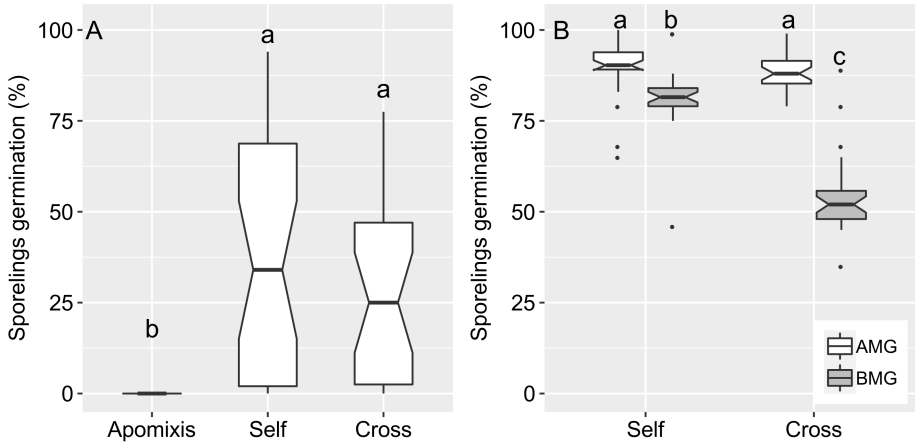

Figure 3. The two series of experiments to examine sexual reproduction in *Isoëtes cangae* and *Isoëtes serracarajensis*. (**A**) Percentage of spore germinations in apomixis (only megaspores, n = 730 megaspores), crosses (n = 1480), and self-fertilization (n = 1310). Each point corresponds to one biological replicate with several megaspores (15–100), and the different symbols represent the four experiments of the first series. Detailed information in Table S1. (**B**) Percentage of *I. cangae* sporelings produced in the self-fertilization and cross-fertilization tests by adding microspores before megaspore germination (BMG) or after megaspore germination (AMG). Each point corresponds to one biological replicate with 10 megaspores (total of 300 megaspores per treatment). Boxes carrying the same letters indicate no significant differences between the treatments after a post-hoc Dunn's test at $p < 0.05$.

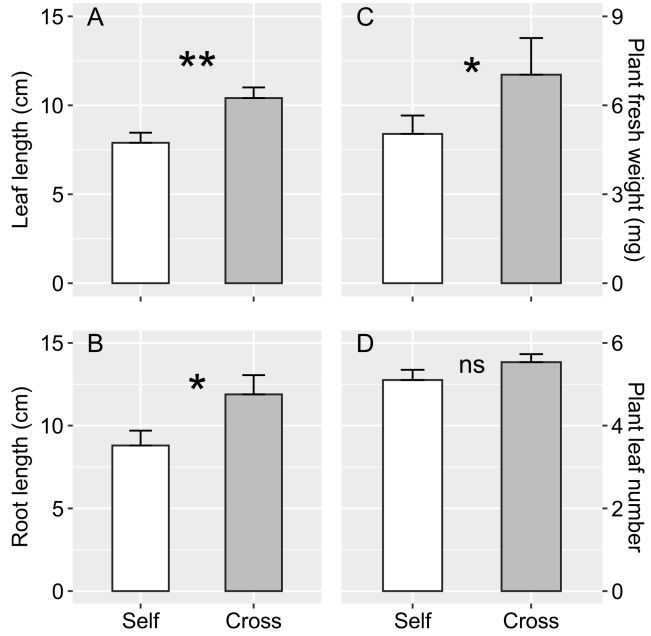

Figure 4. Sporelings growth traits of *Isoëtes cangae* generated by self-fertilization and cross-fertilization. Leaf length (**A**), root length (**B**), plant fresh weight (**C**), and plant leaf number (**D**) were measured after three months of plant cultivation in controlled conditions (see Material and Methods). Values are presented as the means ± confidence interval at $p < 0.01$ (n = 30). 'ns' represents non-significant; * and ** represent significant difference after Student's *t*-test at *p*-values of 0.05 and 0.01, respectively.

3.3. Isoëtes cangae Ex Situ Growth and Sporangia Production

Sporophytes of *I. cangae* were able to reach the adult/reproductive phase before 12 months when cultivated in controlled environmental conditions. We observed the appearance of the first sporangia since the seventh month after planting for the more

vigorous plants. Large variability in sporangia production (Figure 5A) was observed for 12-month-old plants, with a positive relationship to the plant leaf number (Figure 5B). Marked by a substantial difference between substrates, plant growth and sporangia production were higher when sporophytes were cultivated in the presence of lake sediments. During this first year of cultivation, we did not observe the emergence of new sporophytes. They started showing up late in the second year together with visual leaf aging, which developed a yellowish/brownish color in the more external leaves (Figure 5C), suggesting spore release.

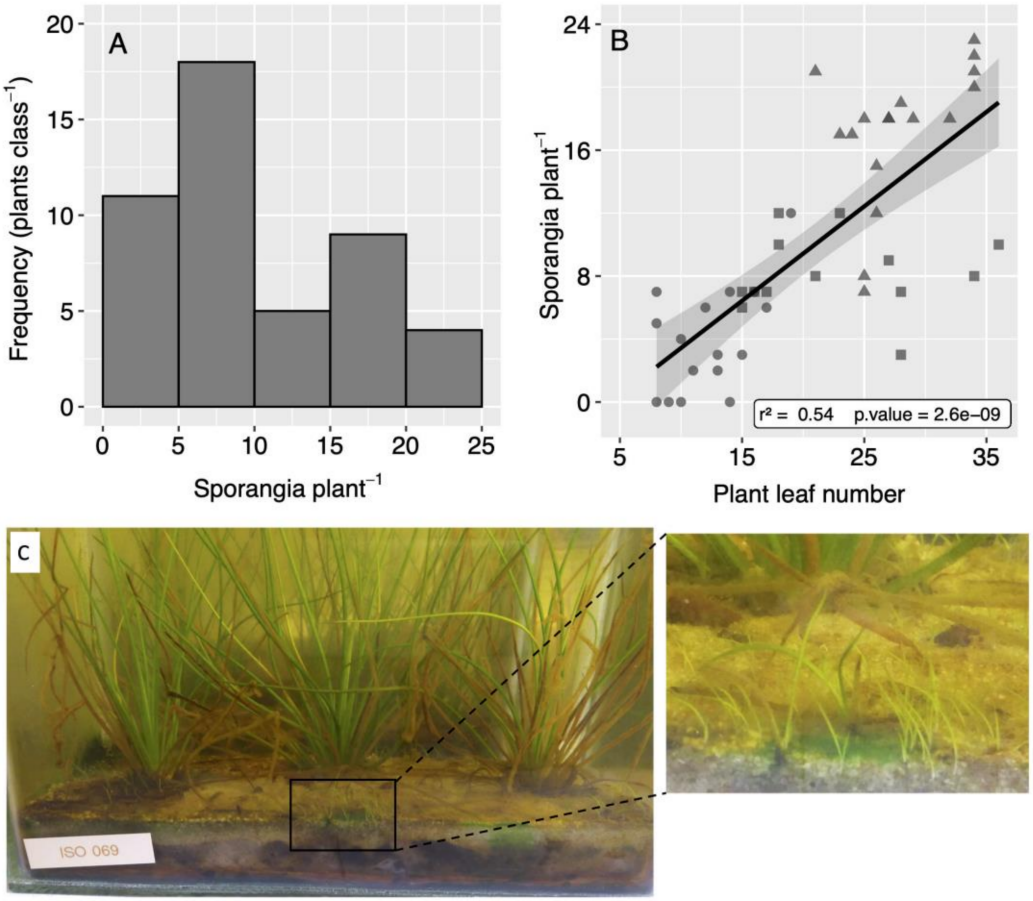

Figure 5. Sporangia and sporophyte production for *Isoëtes cangae* plants growing in controlled environmental conditions. (**A**) Classes of sporangia produced by 12-month-old plants of *I. cangae*, (**B**) a linear relationship between sporangia and plant leaf number (y = 0.615x − 3.093), and (**C**) plants of *I. cangae* 24 months old with several young sporophytes growing close to the mother plants. In (**B**), each symbol represents one plant cultivated in commercial substrate Jiffy-7® (circles), a mixture of Jiffy-7® and lake sediment (squares), and lake sediment (triangles).

After 12 months of growing under greenhouse conditions, a large number of adult plants collected in the field (80%) produced new sporangia. Under the same environmental conditions, 70% of young plants reached the adult phase (producing sporangia). Nonetheless, only 5% of the 220 plants evaluated generated new sporophytes (Figure S2). Once they were isolated in beakers, we attribute their type of reproduction to self-fertilization. The percentage of polymorphic *loci* ranged from 44%. All ISSR analyses confirmed that *I. cangae* is an outcrosser.

3.4. Vegetative or Asexual Reproduction

While we did not observe the appearance of new sporophytes attached to the mother plants in *I. cangae*, they were commonly found attached to the corm of *I. serracarajensis*

(Figures 6A and S3) during the first series of experiments. After 12 months in controlled conditions, 88% (22 out of 25) of *I. serracarajensis* sporophytes produced a mean of four new plants by tillering. The three sporophytes not tillering produced a larger number of sporangia, suggesting a negative relationship between tillering and sporangia production (Figure 6B). Some of the plants showing vegetative propagation did not develop sporangia. Additionally, unlike the observations for *I. cangae*, in *I. serracarajensis,* we did not obtain a significant correlation between sporangia and plant leaf number (Figure 6C), tillering rate or whole plant leaf number (Figure 6D).

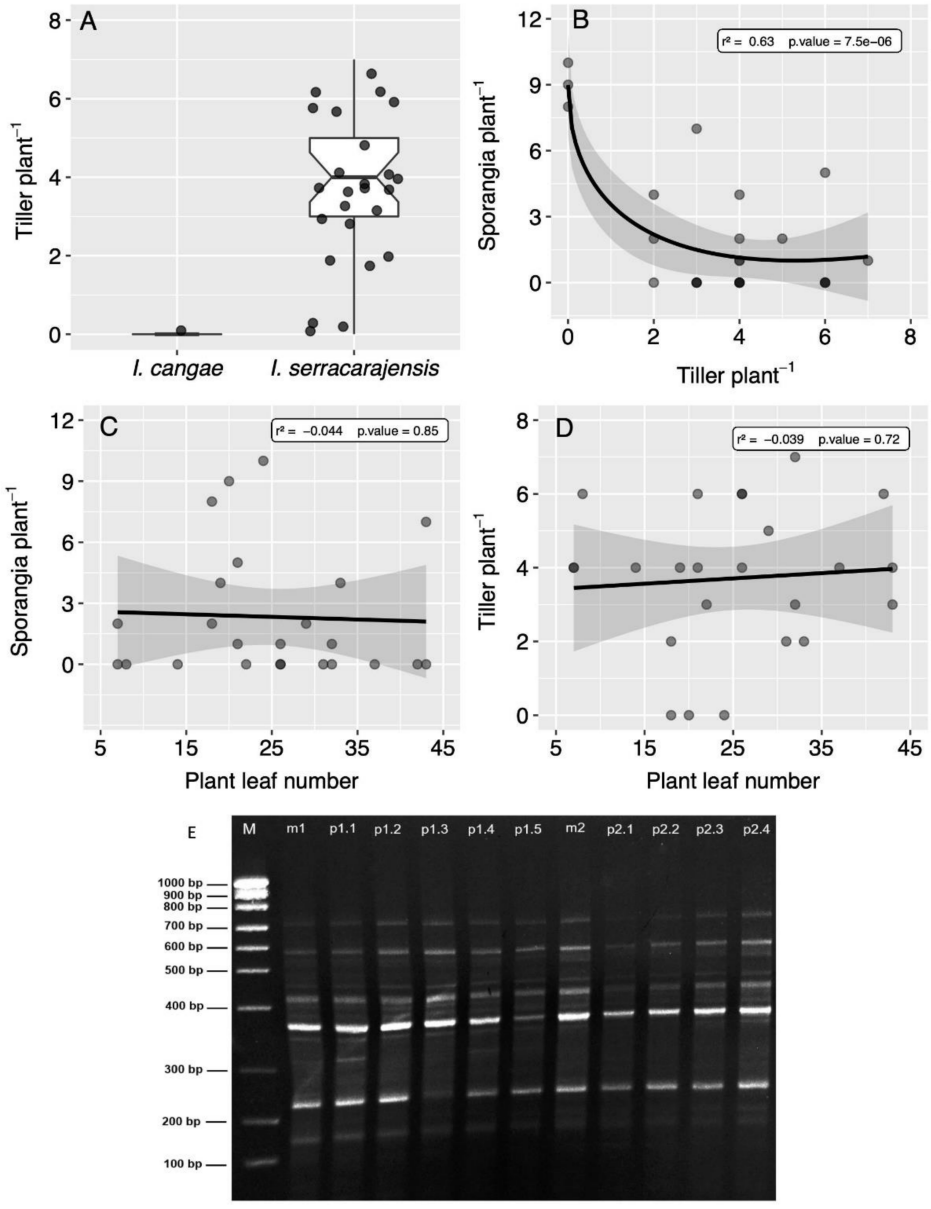

Figure 6. The tillering rate of 12-month-old plants of *Isoëtes cangae* and *Isoëtes serracajensis* (**A**) and the relationship between sporangia and tillers per plant (**B**, $y = -6.9x^{0.5} + 1.51x + 8.98$), sporangia and leaf number (**C**, $y = -0.013x + 2.65$, nonsignificant model) and tiller and plant leaf number (**D**, $y = 0.014x + 3.35$, nonsignificant model). (**E**) Gel electrophoresis pattern of ISSR amplification. M: Size marker; m1: mother plant (1); p1.1 to p1.5 refer to progenes of the mother (1); m2: mother plant (2) (related to m1); p2.1 to p2.4 refer to progenes of the mother (2).

In the second series of experiments, *I. serracarajensis* produced a high percentage of reproductive structures (sporangia) in a greenhouse (70% of the cultivated plants). Half

of the mother plants reproduced and generated three plants each (±2) after 6 months (Figure S2), and after one year, all plants produced progenies. The genetic study confirmed that adult plants of *I. serracarajensis* reproduced by asexual reproduction once the progenies showed multilocus genotypes identical to the mothers (Figure 6E).

3.5. Sporophytes Survival

A high percentage of sporophyte survival was obtained under controlled environmental conditions. In *I. cangae*, more than 98% of young plants survived after being propagated and grown ex situ (laboratory). While lower than that *I. cangae*, the survival rate of *I. serracarajensis* tillers reached 90% (Figure 7).

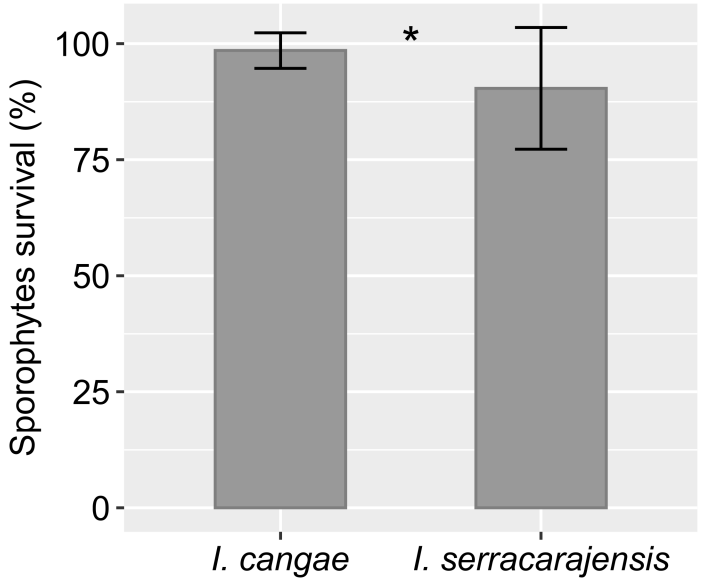

Figure 7. The sporophyte survival of *Isoëtes cangae* and *Isoëtes serracajensis*. The error bar represents the standard deviation, and * indicates significant differences by Mann–Whitney U test at $p < 0.05$.

4. Discussion

Our results suggest that the two endemic and rare *Isoëtes* species from Serra dos Carajás show distinct reproductive strategies: *I. cangae* reproduces by spore germination (sexual propagation) and *I. serracarajensis* reproduces by emitting tillers from the plant corm (vegetative propagation). Sexual propagation has been reported as a common strategy in the *Isoëtes* genus [13,15,17,20] and was previously described as occurring in *I. cangae* [14]. We found that *I. cangae* is able to reproduce via self- and cross-fertilization, excluding the occurrence of vegetative propagation and apomixis. The few individuals obtained from megaspore isolation in this species can be attributed to possible fertilization before sporangia collection, as indicated by ISSR analysis. Otherwise, apomixis should be considered a rare event in *I. cangae*. In fact, apomixis is still rarely reported for *Isoëtes* L. but is fundamental in the propagation of *Isoëtes andicola* [18].

In this study, we obtained a wide range of variations in sporophyte regeneration from in vitro fertilization assays. Higher rates of sporophyte regeneration have been observed for other species, such as *Isoëtes malinverniana* [24], *Isoëtes lacustris* [17], and *Isoëtes sabatiana* [25], and have been attributed to the use of mature reproductive structures, while immature spores could lead to less effective sporophyte regeneration [15,25] or even to nongermination, as previously reported for *I. cangae* [14]. Consequently, the lower sporophyte regeneration obtained in some of our experiments could be affected by the use of spores that were not completely developed. Despite their brownish color at sampling, the sporangia were collected mostly from cultivated plants less than 24 months old. Nonetheless, we cannot exclude the hypothesis that for *I. serracarajensis*, since this

species inhabits seasonal ponds, it has only a few months (the rainy season) in which to grow and reproduce. In that case, the sporangia were sampled from 12-month-old plants.

The sexual propagation of *I. cangae* is an important way to expand its genetic diversity and reduce the risks associated with inbreeding. In recent studies carried out with ISSR markers [16] and a large number of SNPs [26], it was revealed that *I. cangae* consists of a single panmictic population with a moderate levels of genetic diversity and no inbreeding signal. Such diversity is likely to result from the male gamete [27] and young sporophytes movements in the Amendoim lake [28], increasing the possibility of cross-fertilization. As observed in this study, such sporophytes showed a faster leaf and root initial growth. Furthermore, the success of conservation plans is based on preserving the genetic diversity of related species. However, the proximity of male and female sporangia and the gradual release of spores through the decomposition of sporangium tissue increases the chances of self-fertilization in *Isoëtes* [29,30], especially in areas of standing water, such as ponds and swamps, as described by [31] for the species *I. hypsophila*.

The vegetative or asexual reproduction observed in *I. serracarajensis* is probably a less common method reported for the *Isoëtes* genus, although it has been described as the main reproductive strategy for species such as *Isoëtes savatieri* and *Isoëtes chubutiana* [4]. As observed for *I. serracarajensis*, these two species are also able to produce megaspores and microspores, although their reproduction is still linked to tiller bud development from the plant corm. As reported for other related species [32–34], this reproductive strategy can be advantageous for *I. serracarajensis* colonizing adverse environments such as the temporary ponds of Serra dos Carajás, where the short period with available water (five to six months) would not be enough to achieve sexual propagation, i.e., not enough time for young sporophytes to grow and disperse their reproductive structures regenerating new plants able to face severe drought in the region [8,28]. In fact, unlike *I. cangae*, which remains underwater throughout the year and faces less pronounced environmental changes [28], *I. serracarajensis* has been observed only during the rainy season (November to March). Field observations suggest that water shortage is accompanied by leaf drying and fall, while the plant corm remains protected and alive in the soil until the beginning of the next rainy season, when growth resumes (Figure 1). However, we cannot rule out the possibility that plants of *I. serracarajensis* two years old or older may indeed produce substantial reproductive structures during the rainy season. Then, desiccation and refilling of the temporary ponds may trigger spore germination so that sexual reproduction becomes synchronized with favorable environmental conditions. Interestingly, plants of *I. serracarajensis* remained alive and kept green leaves throughout the 12 months in the presence of available water, suggesting that such species can show continuous growth once in favorable environments.

Although *I. cangae* does not face strong environmental seasonality as *I. serracarajensis* does, we did observe a significant difference in sporophyte regeneration from spores collected in field-growing adult plants during the rainy and dry seasons. Marked variability in spore germination has been described for other species, such as *I. lacustris*, when changes in temperature trigger germination and growth [17]. Overall, it has been reported that fine-tuning synchrony with environmental conditions could be advantageous for plant performance under both stressed and nonstressed conditions [35]. Therefore, this synchrony with environmental conditions is still an important trait conserved by both *I. cangae* and *I. serracarajensis*.

Sporophytes of *I. cangae* can produce a large number of viable sporangia when they reach the adult phase growing in favorable conditions, showing a general tendency of larger mother plants producing larger amounts of offspring. Under nonstressing environmental conditions, it is well established that plants developing larger leaf areas to improve their light interception capacity and carbon fixation as well, developing higher yields and seed production [36,37]. However, this pattern was not clearly observed in *I. serracarajensis*, as no correlation between plant size and tillering rate or the number of sporangia was found. Nevertheless, a dichotomy was observed between tillering rate and sporangia production. Their distinct reproductive strategy notwithstanding, both species (*I. cangae*

and *I. serracarajensis*) showed successful sporophyte survival. Such successful propagation and growth, previously reported for the earlier developmental phases of *I. cangae* [14], were confirmed here for this species and extended for *I. serracarajensis*, reinforcing that both species are resilient and able to grow and thrive ex situ and possibly in other environments than those currently occupied.

In this study, we outlined the distinct reproductive strategy of the two endemic *Isoëtes* species of Serra dos Carajás and attributed that difference to the contrasting habitat occupied by each of them. We improved the available knowledge of the reproductive biology of both species, showing that sporophytes of *I. cangae* can be propagated in larger numbers from mature plant spores, requiring the release of the male gamete to fecundate the megaspore, while *I. serracarajensis* displays vegetative propagation by developing new sporophytes from the plant corm. We also highlighted details of plant growth and development and the relationship between plant growth and reproductive traits. Finally, we demonstrated that both species could produce sporophytes with a high survival rate. Together, these results are of paramount importance to establish conservation plans for both species, considering the advantages of sexual propagation to maintain the genetic diversity of *I. cangae* and the diligent management required to do the same with asexually propagated *I. serracarajensis*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13080348/s1. Table S1: Experimental setup applied to examine the sexual reproduction of two *Isoëtes* L. species endemic to Serra dos Carajás/Brazil. Table S2: Description of ISSR polymorphic primers validated for the genus *Isoëtes* L. Figure S1: A subset of *Isoëtes cangae* germinated megaspores from apomixis treatment (isolated for 15 days after rhizoid emission and archegonia appearance) that developed into sporophytes a few days after microspore addition. Figure S2: Mother plants and their respective sporophytes of *Isoëtes cangae* (a) and *Isoëtes serracarajensis* (b) reproduced in greenhouse conditions. Figure S3: The vegetative reproduction of *Isoëtes serracarajensis*; (a) and (b) new sporophytes (tillers) attached to the mother plant that can easily be detached; (c) and (d) another adult plant and its respective tillers; (e) details of root emission from the new corm fragment at the leaf base.

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