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CELLULAR AND MOLECULAR BIOLOGY

Further evidence for the hybrid status of the Brazilian native fern *Hypolepis ×paulistana* (Dennstaedtiaceae)

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Abstract: *Hypolepis ×paulistana* was described in 2016 as a putative hybrid, known from a single gathering. The hybrid status of these plants was based solely on the intermediate morphology of the sporophyte, when compared to its presumed parent species. These were thought to be *H. stolonifera* and *H. rugosula*, but, *H. rigescens* (Kunze) T. Moore could not be explicitly ruled out either. In the present work, we tested the hybrid status of *Hypolepis ×paulistana* adding palynological evidence and by using chloroplast sequences to unambiguously identify the maternal progenitor of the species. We find that sporangia of *Hypolepis ×paulistana* contain both well-formed spores, as well as spores with morphological and developmental anomalies. The size of the regular spores and the abnormal spores suggest that *H. ×paulistana* is likely a diploid, and probably infertile hybrid. The ornamentation of the regular spores of *H. ×paulistana* is similar to that of *H. stolonifera*. The chloroplast sequences of *H. ×paulistana* are identical to those of *H. stolonifera,* as well as their sister position within the global phylogeny of the genus. Thus, we provide new evidence for the hybrid status of *H. ×paulistana*, and we corroborate the earlier finding that *H. stolonifera* is the maternal parent.

Key words: Atlantic Forest, chloroplast markers, hybridization, palynology, phylogeny, southeastern Brazil.

INTRODUCTION

Hypolepis Bernh. (Dennstaedtiaceae) is a subcosmopolitan, monophyletic, fern genus with about 80 species, mostly distributed in the Neotropics and Oceania/east Asia (Brownsey 1987, Xing et al. 2013, Schwartsburd & Prado 2015, Schwartsburd et al. 2020). Some species of *Hypolepis* are difficult to circumscribe, and this may be due to events of hybridization, aneuploidy, and polyploidy which are common in the genus (Brownsey 1983, Brownsey & Chinnock 1984). In Australia and New Zealand, several cases of hybridization have been reported, and corroborated through combinations of intermediate morphology of specimens (Carse 1929, Cockayne & Allan 1934, Brownsey & Chinnock 1984), anomalous or aborted spores (Brownsey & Chinnock 1984), and chromosome counting (Manton & Sledge 1954, Brownsey & Chinnok 1984). In South America, some hybrids have also been reported based on morphology (Schwartsburd & Prado 2015, 2016) and chromosome counting (Smith & Mickel 1977).

Recently, Schwartsburd et al. (2020) presented evidence that the spore length in *Hypolepis* is directly related to the ploidy of the taxa, and that diploid and tetraploid status can be assigned using these measurements. They suggested that combinations of sporophyte morphology, palynology, chromosome counts and chloroplast DNA sequences, have the potential to unambiguously reveal diploid

and tetraploid hybrids (or, tetraploid species with hybrid origin), as well as their maternal inheritance. Based on the combination of these traits, the authors proposed an allotetraploid origin for six species (or, tetraploid hybrids) and two diploid hybrids, one of which is infertile (Schwartsburd et al. 2020: Table II).

Hybridization is common in ferns (Barrington et al. 1989) and is thought to be one of the major processes in their evolution (Knobloch 1996, Sigel 2016). Although palynological studies of hybrid ferns are still scarce, the presence of anomalous reproductive characters, in combination with morphological intermediacy, is a strong evidence of hybridization events. In this sense, fern hybridization is often associated with failure of indusium eversion; collapsed, small, unopened sporangia; alterations of sporoderm and protoplast developments (Wagner et al. 1986, Barrington et al. 1989); the presence of blackened materials on the inner surfaces of the sporangial capsules (Wagner et al. 1973); abnormal spore shapes, such as spherical, twisted, square, and/or triangular, and spores with different sizes mixed in the same specimens (Wagner & Chen 1965, Wagner 1973) and variations in the ornamentation, pigmentation, degree of development and form of dispersion (through diads or tetrads) (Morzenti 1966, Wagner & Boydston 1958, Morbelli 1974, Hennipman 1977, Wagner 1980, Sleep 2014).

Additionally, the presence of aborted spores in hybrids is an indicator that they are not viable (Manton 1950, Wagner 1969) and, therefore, that the hybrids which produce them are sterile (Wagner & Darling 1957, Wagner 1971, Chang et al. 2009). According to Wagner (1974) the finding of specimens that produce aborted spores is frequently correlated with the sympatric appearance of two closely related species that are capable of forming hybrids. For these authors, the origin of the anomalies could be due to accumulated genetic differences between the parents and presumably sterility barriers. However, there are numerous examples of hybrid spores with the ability to germinate and form gametophytes (Morzenti 1962, Mayer & Mesler 1993), and meiotic mechanisms that allow the fertility of such hybrids.

Concerning to the identification of parental species, it is known that chloroplast and mitochondrial DNAs are maternally inherited in ferns (i.e., from the egg-cell), whereas the paternal inheritance (the atherozoid) contributes only to nuclear DNA (Gastony & Yatskievych 1992, Kuo et al. 2018). And so, in hybridization events, the hybrids would have the same chloroplast and mitochondrial DNAs as their maternal progenitors (Vogel et al. 1998, Xiang et al. 2000, Testo et al. 2015, Hornych et al. 2019). On the other hand, the identification of paternal lineages requires more complex analyses, such as combining sequences of nuclear markers with sequences of low-copy markers (e.g., Pereira et al. 2019).

In Brazil, two *Hypolepis* hybrids have been proposed (Schwartsburd & Prado 2015, 2016, Schwartsburd et al. 2020). One of these was named *H. ×paulistana* Schwartsb. & J. Prado and it was suggested to be a hybrid based on the intermediate morphology between it and the two suggested parents: *H. stolonifera* Fée, and *H. rugosula* J. Sm. subsp. *pradoana* Schwartsb. (Schwartsburd & Prado 2016). *Hypolepis stonolifera* was further implicated because it was found growing near the hybrid in the type locality of *Hypolepis ×paulistana*. Furthermore, another similar species which could potentially be interpreted as a parent is *H. rigescens* because, besides *H. rugosula* and *Hypolepis ×paulistana*, they are the only *Hypolepis* taxa with glandular hairs in southeastern Brazil.

Regarding their geographic distributions, *H. stolonifera* is a common widespread species

that is found in habitats similar to that of the type locality; *H. rugosula* subsp. *pradoana* is comparatively rare, restricted to the highlands of the Atlantic Forest, and expected to occur at higher elevations than the type locality (from 2000 m to 2600 m); and *H. rigescens* occurs in lower elevations in southeastern Brazil, around 600 to 1200 m. (Schwartsburd & Prado 2015, 2016).

Additional *Hypolepis* spp. occurring in Brazil (*H. acantha* Schwarstb., *H. miodelii* Schwartsb., *H. mitis* Kunze ex Kuhn, and *H. repens* (L.) C. Presl), can be excluded because they show clear morphological differences with respect to *H. ×paulistana*—their leaves are much larger, they are aculeate, and they have sparse indument (Schwartsburd & Prado 2015, 2016).

Phylogenetic inference can also be used to infer parentage of the presumed hybrid. Schwartsburd et al. (2020) found that *H. ×paulistana* is nested within the *Hypolepis stolonifera*-clade, among specimens of *H. stolonifera, H. acantha* Schwartsb., *H. grandis* Lellinger, and another hybrid involving *H. stolonifera* (*H. mitis × H. stolonifera*). *Hypolepis rigescens* is nested within the *Hypolepis repens*-clade, an essentially western South American/Mesoamerican clade of *Hypolepis*. The *Hypolepis stolonifera*-clade and the *Hypolepis repens*-clade are sister clades.

On the other hand, *H. rugosula* subsp. *pradoana* Schwartsb. nested within the *Hypolepis rugosula*-clade, among specimens of *H. rugosula* from elsewhere (Australia, Chile, New Zealand, South Africa, etc.), far removed from the *Hypolepis stolonifera*-clade.

In the present work, we aimed to test the hybrid status of *H. ×paulistana,* adding palynological studies to the previous morphological hypothesis, and studying the chloroplast sequences to test the putative maternal progenitor of the species.

MATERIALS AND METHODS

Examined specimens

Specimens of *Hypolepis ×paulistana* were collected in January of 2010, along the road to Pico do Itapeva, a highland mountain area of around 1880 m located in Pindamonhangaba, state of São Paulo, Brazil, within the Atlantic Forest biome (type locality) and deposited in herbaria of Duke University (DUKE), La Plata Museum (LP), Missouri Botanical Garden (MO), Jardim Botânico do Rio de Janeiro (RB), Instituto de Botânica (SP), and Universidade Federal de Viçosa (VIC). The letters MP, in the specimen investigated indicate the reference number of palynological sample filed in the Laboratory of Palynology, Faculty of Natural Sciences and Museum (La Plata, Argentina).

We also conducted searches for additional herbarium specimens of *Hypolepis ×paulistana*; these are listed in Schwartsburd & Prado (2016).

Searches for specimens of potential parent species: *H. rigescens* (Kunze) T. Moore, *H. rugosula,* and *H. stolonifera*, were conducted in LP, PACA, SP, VIC and VT. These specimens are listed in Table III.

Morphological comparisons

General morphological comparisons of laminae characters were carried out among *Hypolepis ×paulistana, H. rigescens*, *H. rugosula,* and *H. stolonifera*. We used standard terminology for the morphological descriptions (Lellinger 2002).

Palynological analysis

Spores were studied using Light microscope (LM) and Scanning electron microscope (SEM). For LM, spores were studied without chemical treatment since the perispore does not resist acetolysis treatment (Erdtman 1960). Spores were mounted in gelatin glycerin jelly. For normal spores of *Hypolepis ×paulistana*, polar

diameter, major and minor equatorial diameters (Ramos Giacosa et al. 2009, Figure 1), perispore and exospore thickness were measured (Nayar 1964). Likewise, spores with abnormalities found for this species were identified and measured when possible. To calculate the percentage of normal spores and spores with anomalies, we randomly selected a total of 1654 spores from different mature sporangia. The observations were performed with Olympus BH2 LM and photographs were taken with a Nikon Coolpix S10 digital camera at the Palynology lab from Faculty of Natural Sciences and Museum (La Plata, Argentina).

For SEM observation spores were treated with hot 3% sodium carbonate, washed, dehydrated, suspended in 96% ethanol and then transferred to acetate plates (Morbelli 1980). After drying, the spores were coated with gold. The observations were performed with a Jeol JSMT100 from the Microscopy Service of Faculty of Natural Sciences and Museum (La Plata, Argentina) and with a Philips XL 30 TMP New Look from the Microscopy Service of Natural Science Argentine Museum "Bernardino Rivadavia" (Buenos Aires City, Argentina).

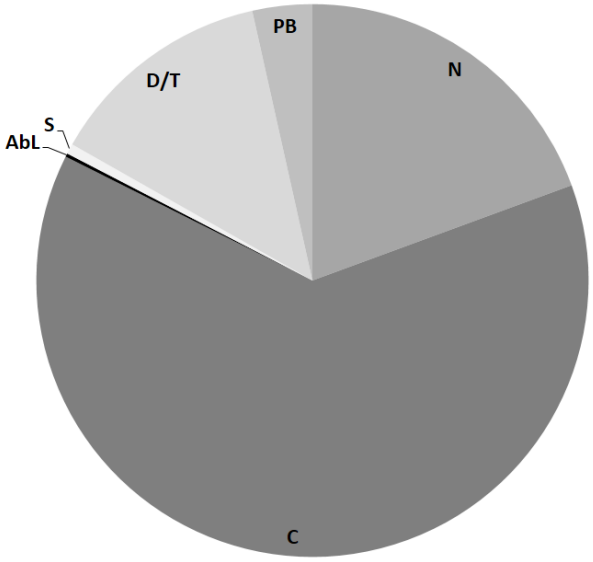

Figure 1. Variability of *Hypolepis x paulistana* spores. AbL= laesurae with abnormalities (0.2%), C = Collapsed spores (63%), D/T = Dyads or Tetrads (13.3%), N = Normal spores (19.4%), PB = Bodies of perisporic material (3.5%), S = Spheroidal spores (0.6%).

Kremp (1965), Huang (1981), Tryon & Lugardon (1991), Punt et al. (1994, 2007), Lellinger (2002), and Sáenz Laín (2004) were followed to describe the spore morphology and their wall ultra–structure. To describe the ornamentation elements of *Hypolepis ×paulistana* and *H. stolonifera* the concept of "knife" was introduced, which is a flattened element three times longer than wide (3:1) (Figure 3b). The elements that constitute the ornamentation of these species were previously described by Tryon & Lugardon (1991) as "echinae". However, we consider it necessary to introduce a new term to distinguish these elements, because the authors described echinae as being radially symmetrical, which is not observed in the flattened elements of the studied species ornamentation.

cpDNA comparison and phylogenetic analyses

For comparisons of chloroplast DNA, we use at the results of Schwartsburd et al. (2020), in which a Maximum Likelihood, phylogenetic tree was inferred from five markers (*atpA, rbcL, rpl16, rps4-trnS, trnL-trnF*), from nearly half of all *Hypolepis* species Worldwide. Having that tree in mind, we downloaded their generated sequences now available on GenBank (https://www.ncbi.nlm.nih.gov/genbank/). We compared the sequences of three markers (*rpl16, rps4-trnS, trnL-trnF*) of one specimen of *H. ×paulistana* with two of *H. stolonifera*, one of *H. rugosula* and one of *H. rigescens* using an alignment generated with MAFFT (Katoh & Standley 2013) in Geneious Prime®, version 2019.0.4 (Biomatters Ltd., San Francisco, California, USA).

RESULTS

Morphological comparisons and geographic distributions

Table I shows morphological comparisons and geographic distributions of the studied species.

Palynological results

Hypolepis ×paulistana Schwartsb. & J. Prado

Spores are monolete, bilateral, elliptic in polar view (Figure 2a). Spore diameters are shown in Table II. In equatorial view, the proximal face is flat and the distal face is convex to hemispheric (Figure 2b). The laesurae extends from 2/3 to 3/4 of the length of the spore. With LM, the perispore is light brown and the exospore is hyaline.

The ornamentation has short knife and echinae 1–5.03 µm long, distributed throughout the surface (Figure 3a–b). The threads are 0.09–3.5 nm thick, connect the ornamentation elements, and form a network (Figure 3a). The surface is microverrucated (Figure 3b)

In addition to the "normal" looking spores, abnormal spores with alterations related to shape, dimensions, grouping, and form of the laesura (Figure 2c, 3c) were found within the sporangia:

1. Collapsed spores without protoplast (Figure 2c–d, 3c–d): Spores with major equatorial diameter of 20–31 µm forming major concavities or twisting, like a prune. Some of them do not have protoplast (black spores in Figure 2c–d). Spores without ornamentation can be observed.

2. Abnormalities or absence of laesurae: There are spores with curved laesurae, that extend beyond the proximal face (Figure 2e). Also, spheroidal spores without lesura were observed (Figure 2f).

3. Spheroidal spores (Figure 2f, 3e): Spores of radiated symmetry, 30–32 µm diameter, alete, with similar ornamentation to normal spores were observed.

4. Dyads and tetrads (Figure 2g–h, 3d, f): groups of two or four mature and immature spores with different shapes are seen.

Table I. Morphological and habitat comparisons among *Hypolepis ×paulistana* and the probable parents.

Character/taxon	*H. stolonifera*	*H. ×paulistana*	*H. rugosula*	*H. rigescens*
Leaf length	1.2–2 m	0.5–1 m	0.15–1.2 m	1–2.3 m
Petiole color	Golden brown	Light brown	Burgundy	Golden brown
Aculei on petioles	absent	absent	absent	present
Shape of basal pair of pinnae	Inequilateral	Sub-equilateral (intermediate condition)	Equilateral	Sub-equilateral
Indument	Catenate-acicular hairs	Catenate-acicular and catenate-glandular hairs	Catenate-acicular and catenate-glandular hairs	Catenate-acicular and catenate-glandular hairs
Sorus position	Marginal	Marginal	Sub-marginal	Marginal
Adaxial indusium	Developed and ciliate	Developed and ciliate	Undeveloped	Developed, not ciliate
Habitat elevation in SE Brazil	1200–2250 m	1880 m	2000–2600 m	600–1200 m
Phylogenetic position	*Hypolepis stolonifera*-clade	*Hypolepis stolonifera*-clade	*Hypolepis rugosula*-clade	*Hypolepis repens*-clade

5. Bodies of perisporic material (Figure 2i): there are small amorphous bodies of different size between spores, with a similar color and ornamentation to peripore.

Among the total of studied spores of *Hypolepis ×paulistana*, 19.4% were "normal" and 80.6% showed one of these morphological and developmental anomalies (Figure 1).

Hypolepis rugosula subsp. pradoana Schwartsb.

Spores are monolete, bilateral, oblong to sub–elliptic in polar view (Figure 4a). Spores diameters are shown in Table II. In equatorial view the proximal face is concave to plane and the distal face is hemispheric (Figure 4b). The laesurae is straight and extends from 2/3 to 3/4 of the length of the spore. The perispore is hyaline and the exospore is light brown at LM. The ornamentation is cristate. The crests are variable in length and height, with an irregular margin.

Hypolepis stolonifera Feé var. stolonifera

Spores are monolete, bilateral, elliptic in polar view and plane-hemispheric in equatorial view (Figure 4c–d). Spores diameters are shown in Table II. The laesurae extends from 3/4 of the length of the spore. Perispore is light brown and the exospore is yellowish to hyaline with LM. Spore ornamentation is formed by echinae and knife elements of 2.1-3.5 µm long, distributed throughout on the surface.

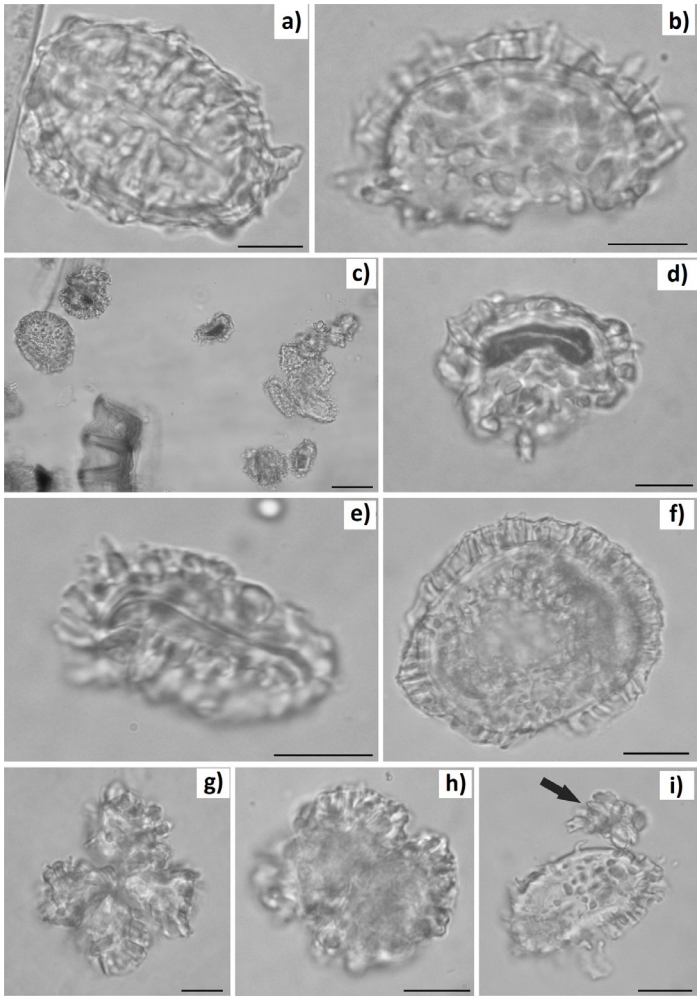

Figure 2. Spores of *Hypolepis x paulistana*, with LM. a) Normal spore in proximal view. b) Normal spore in equatorial view. c) Spores with abnormalities. The dimensions of the spores vary within the same specimen and have irregular shapes. d) Collapsed spore with concavities and without protoplast. e) Spore with laesura courved at ends. f) Spheroidal spores with radiated symmetry. g) Tetrads of spores. h) Dyads of spores. i) Amorphous body ornamented by knife elements similar to those of the normal spores perispore (arrow). Scale bars c = 30 µm; a–b, d–i = 10 µm.

Table II. Spore diameters of *H. ×paulistana* and potential parent species, *Hypolepis rugosula* subsp. *pradoana* and *H. stolonifera* var. *stolonifera*. Values expressed in microns. μ = mean, s = standard deviation.

		Hypolepis rugosula subsp. pradoana	*H. ×paulistana*	*H. stolonifera var. stolonifera*
Polar Diameter		27–34	20–31	21–27
	μ	29.92	26.38	23.58
	s	2.26	5.08	2.05
Mayor Equatorial Diameter		45–56	22–48	25–42
	μ	50.67	35.44	36.08
	s	3.05	6.05	2.26
Minor Equatorial Diameter		26–35	16–32	22–29
	μ	30.17	22.28	24.90
	s	2.56	4.61	3.09
Perispore thick		0.1–8,8	1.1–3.01	0.4–3.7
Exospore thick		0.8–1.2	0.5–1.03	0.1–0.9
Laesurae length		26–40	25–27	14–18

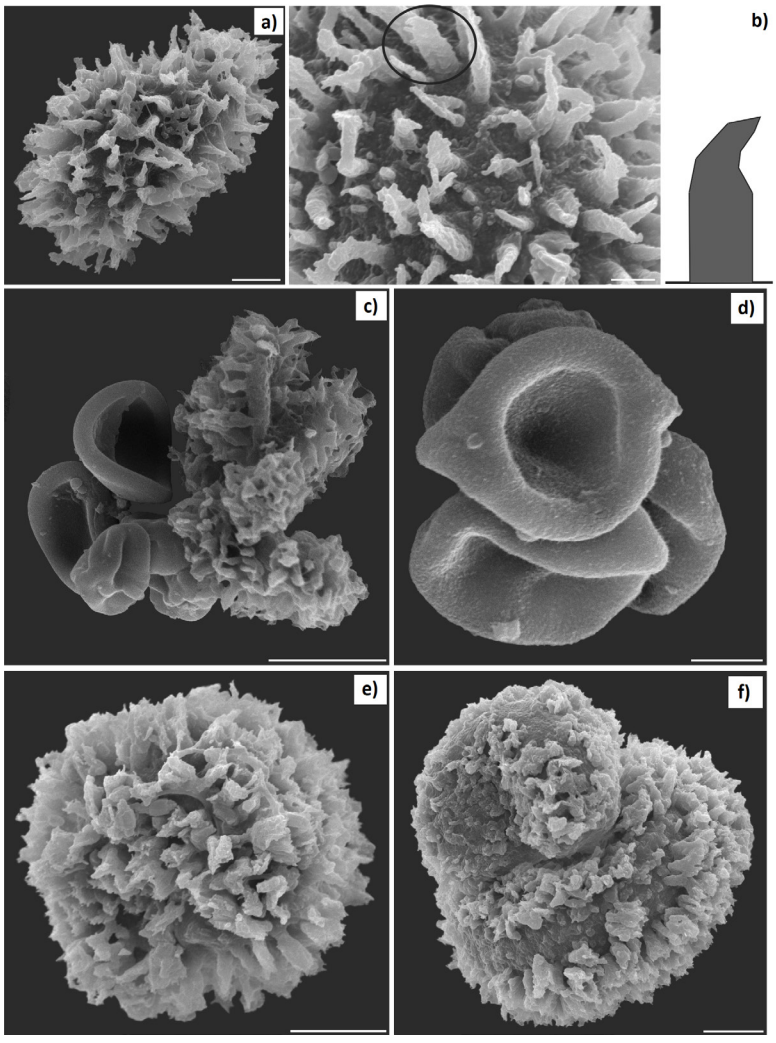

Figure 3. Spores of *Hypolepis x paulistana*, with SEM. **a)** Spore in distal view. **b)** Detail of ornamentation. Circle = Knife element. Scheme = knife element in longitudinal view. **c)** Spores with abnormalities. **d)** Tetrads of collapsed spores without ornamentation. **e)** Spheroidal spores. **f)** Dyad of spores. Scale bars a, e–f= 10 μm; b= 2 μm; c–d= 20 μm.

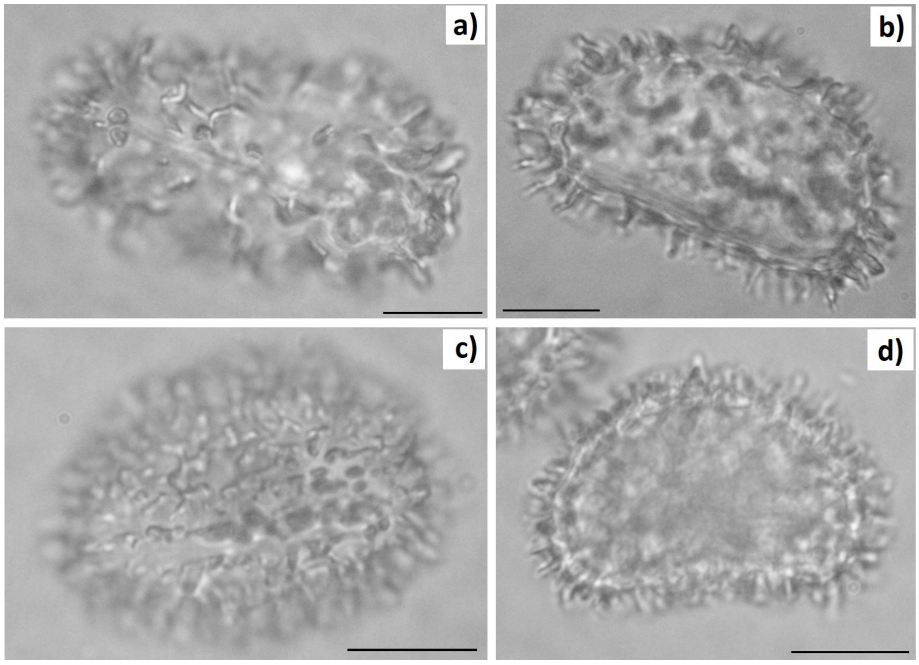

Figure 4. Spores of parent species. **a–b** Spores of *Hypolepis rugosula* subsp. *pradoana*. **a)** Spore in proximal view. **b)** Spore in equatorial view. **c–d.** Spores of *Hypolepis stolonifera* var *stolonifera*. **c)** Spores in proximal view. **d)** Spores in equatorial view. Scale bar = 10 µm.

Table III. Specimens examined of *Hypolepis ×paulistana* potential parent species.

Taxon	Palynological studies	cpDNA comparison			
	Voucher	Voucher	GenBank accession numbers		
			rpl16	rps4-trnS	trnL-trnF
Hypolepis ×paulistana	*Schwartsburd 2298* (LP)	*Schwartsburd 2298* (LP)	MT470063	MT593229	MT593302
Hypolepis rugosula* subsp. *pradoana	*P.B. Schwartsburd & J.B.S. Pereira 2310* (LP)	*P.B. Schwartsburd et al. 4390* (VIC, VT)	MT633769	MT563108	MT593280
		Schwartsburd 4404 (VT)	MT470048	MT563109	MT593281
		Schwartsburd 4404b (VT)	MT470049	MT563110	MT593282
Hypolepis stolonifera* var *stolonifera	*Sehnem 1156* (PACA); Schwartsburd & *A.C. Corazzini 1928* (LP)	*P.B. Schwartsburd & R. Santana 4371* (VIC, VT).	MT470060	MT593227	MT593294
		P.B. Schwartsburd & R. Santana 4372 (VIC, VT).	MT470061	MT563115	MT593295
		Schwartsburd 4420 (VT)	MT470062	MT563116	MT593296
Hypolepis rigescens (var. rigescens)		*Wood 16410* (VT)	MT470046	MT563105	MT593276

Chloroplast DNA comparisons (Figure 5)

In our chloroplast DNA alignment, we found variation in the *rpl16* intron.At positions 315 and 320 in the three *Hypolepis stolonifera* samples and in *H. ×paulistana* there is a thymine, whereas in the three *H. rugosula* and in *H. rigescens* there is a cytosine. In regions 464 and 476 *H. ×paulistana* and *H. stolonifera* share an adenine, whereas the other species have a guanine there. On positions 567 to 570, *H. rugosula* has an insertion of GGAA unique to it.

In our alignment of the intron *rps4-trnS*, *Hypolepis rugosula* has several mutations unique to it, including an insertion of TAAGC at positions 219 to 223. At position 185, *H. ×paulistana* and *H. stolonifera* share a thymine, whereas the other species have a cytosine. At positions 452 to 455, *H. ×paulistana* and *H. stolonifera* share the sequence of four guanines, whereas in *H. rigescens* this sequence is formed by GGAG, and in *H. rugosula*, by TGGA.

Finally, in our intron *trnL-trnF* alignment, *Hypolepis rugosula* has also several unique mutations to it, and two conspicuous insertions at positions 71 to 77 and at 191 to 205. *Hypolepis ×paulistana* and *H. stolonifera* share an insertion of an adenine at position 17 (lacking in the other two species), and a guanine at position 116 (adenine in *H. rigescens*; thymine in *H. rugosula*).

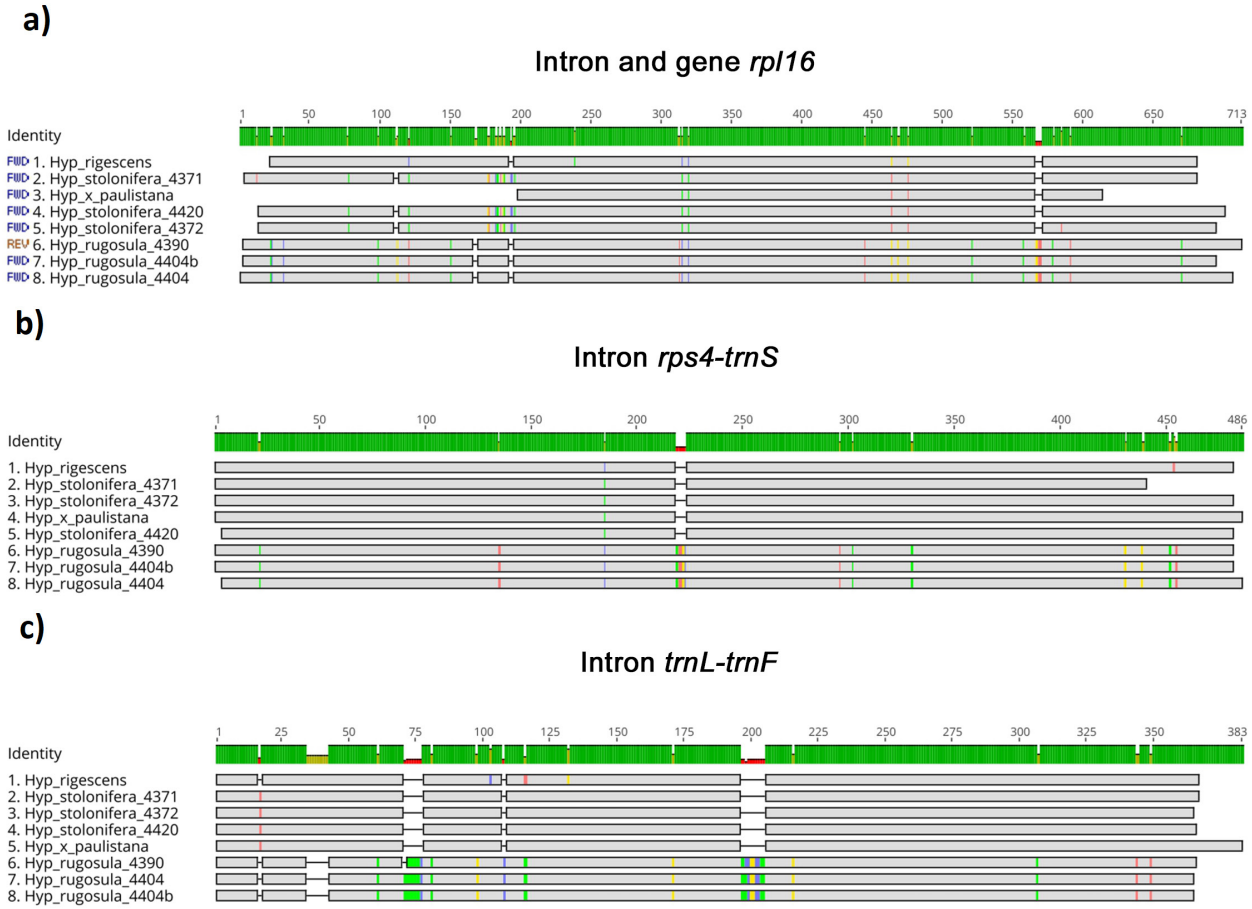

Figure 5. Chloroplast DNA comparison between *Hypolepis ×pauslistana, H. rigescens*, three specimens of *H. rugosula*, and three of *H. stolonifera*. A. intron and gene *rpl16*. B. intron *rps4-trnS*. C. intron *trnL-trnF*. Gray color refer to nucleobases common to all specimens; pink – adenine; blue – cytosine; yellow – guanine; green – thymine.

DISCUSSION AND CONCLUSIONS

The presence of both morphological intermediacy and abnormal spores support the hybrid status of *Hypolepis ×paulistana*. The similar size of the spores in comparison to other diploid species of *Hypolepis* suggests that it is formed from diploid parents. The high percentage of abnormal spores supports the conclusion that *H. ×paulistana* is, infertile, occasional hybrid—perhaps produced only once—and probably incapable of reproducing sexually itself.

The "normal" spores of *Hypolepis ×paulistana* have similar ornamentation compared to *Hypolepis stolonifera*. The surface of spores in both species shows echinae and knife-like elements, distributed at random and connected by branched threads. In relation to the size of the spores, the polar diameter in *H. ×paulistana* is intermediate between *H. stolonifera* and *H. rugosula*, while the equatorial diameters are slightly smaller in the hybrid. The hybrid has the form of distal pole (from convex to subconical) more variable than the parent species.

Different diameters and forms of the *Hypolepis ×paulistana* "abnormal" spores were found, which vary from spherical to collapse. Taylor et al. (1985) and Wagner et al. (1986) have discussed that spores of hybrid origin tend to display high variability in size, shapes, and in the relation between protoplast/wall. These differences, as well as the presence of dyads or tetrads also formed by *Hypolepis ×paulistana*, could be due to an unequal chromosomal number of the sister spores (Wagner & Boydston 1958) and modifications in the meiotic division of the spore stem cells (Hickok & Klekowski 1973). Likewise, alterations in the meiotic process were related to the production of spheroidal "giant spores" (DeBenedictus 1969) as those observed for *Hypolepis ×paulistana*.

Additionally, other traits observed in *Hypolepis ×paulistana* "abnormal" spores were associated with hybridization events, such as massive perisporal development in collapsed spores (Wagner 1968, 1980), laesurae abnormalities (Brown 1960, Erdtman 1958, Erdtman & Praglowski, 1959, Wagner 1974), and small amorphous bodies of perisporic material interspersed within the sporangium (Wagner & Boydston 1958; Wagner & Chen 1965, Wagner et al. 1986)

The diversity of anomalies observed in the spores of *Hypolepis ×paulistana* and the similarities found with previous studies carried out in other taxa provide new evidence about the hybrid origin of the species. Further corroboration from cytological studies would be valuable, since other environmental and genetic factors have been known to cause alterations in the production of spores (Kanamori 1969, Wagner 1974, 1986). Finally, the cpDNA comparison and the phylogenetic position of *H. ×paulistana* alongside *H. stolonifera,* far away from *H. rugosula* and *H. rigescens* are evidences that suggest *H. stolonifera* as the maternal progenitor of *H. ×paulistana*. The paternal inheritance is still only suggested by morphological data (i.e., presence of glandular hairs) and habitat preference (i.e., elevation), pending further molecular investigation.

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