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Original Paper

Embryology and fertility of the natural tetraploid *Lessingianthus plantaginoides* (Asteraceae, Vernonieae): taxonomic implications

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Abstract

Lessingianthus plantaginoides (Vernonieae, Asteraceae) is a small natural tetraploid shrub that inhabits rocky highlands from South America. The population studied inhabits and covers an extensive region of a private reserve with high local biodiversity and animal and plant endemisms. With the purpose of providing insights into the cyto-embryology of this tetraploid species, the aims of this study were: to perform an ontogenetic study of the male and female gametophytes of *L. plantaginoides*; to carry out detailed meiotic analysis and evaluate the fertility of this species; to document and provide highlights on taxonomic implications of their reproductive aspects. *Lessingianthus plantaginoides* presented the following male and female gametophyte traits: dicotyledonous type of anther wall development, tetrahedral tetrads, 3-celled mature pollen grains; development of the chalazal megaspore, monosporic embryo sac and Polygonum type of megagametophyte development. The meiotic behavior was regular, the spores were tetrads of equal size and the pollen grains were highly stainable. *Lessingianthus plantaginoides* is a highly diplodized autotetraploid that reproduces sexually and has high meiotic regularity; which is apparently responsible for its colonization potential. It now seems certain that polyploid speciation plays a significant role in the establishment and diversification of the genus.
Key words: embryology, gametophyte development, polyploidy, Vernonieae.

Resumo

Lessingianthus plantaginoides (Vernonieae, Asteraceae) é um pequeno arbusto com tetraploidia natural que habita as formações rochosas da América do Sul. A população estudada ocorre e se estende por uma região de uma reserva privada com elevada biodiversidade local e endemismos de animais e vegetais. Com o intuito de fornecer conhecimentos sobre a citologia desta espécie tetraploide, realizamos um estudo ontogenético dos gametófitos masculinos e femininos de *L. plantaginoides*; fizemos uma análise meiótica detalhada e avaliamos a fertilidade desta espécie; documentamos e demos destaque às implicações taxonômicas dos seus aspectos reprodutivos. *Lessingianthus plantaginoides* apresentou os seguintes traços dos gametófitos masculinos e femininos: desenvolvimento da parede da antera é do tipo dicotiledôneo, tétrades tetraédricas, grãos de pólen maduros tricelular; desenvolvimento do megásporo calazal, saco embrionário monospórico e desenvolvimento do megagametófito tipo Polygonum. O comportamento meiótico é regular, os esporos são tétrades de igual tamanho e os grãos de pólen são altamente tingíveis. *Lessingianthus plantaginoides* é um autotetraplóide com alta diplodização que se reproduz sexualmente e tem uma elevada regularidade meiótica; o que é aparentemente responsável pelo seu potencial de colonização. Ao que tudo indica a especiação poliplóide desempenha um papel significativo no estabelecimento e diversificação do gênero.
Palavras-chave: embriologia, desenvolvimento de gametófitos, poliploidia, Vernonieae.

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Introduction

Polyploidy, defined as the duplication of the genomic complement, is a form of plant speciation. It was formerly ignored as a dead end, until phylogenetic analyses revealed the contribution of polyploidy to the evolutionary history of the Angiosperms (Soltis *et al.* 2003). Because of this, the research was primarily focused on the origin and maintenance of polyploids (Abbott & Lowe 2004; Schinkel *et al.* 2017; Certner *et al.* 2017; Kovalsky *et al.* 2017), the frequency of recurrent polyploidization (Soltis & Soltis 1999; Soltis & Soltis 2000; Falistocco 2016), the ecological effects of plant polyploidy (Ramsey 2011; Beest *et al.* 2012), and the genetic, epigenetic, chromosomal, and genomic consequences of polyploidization (Comai 2005; Adams & Wendel 2005; Rapp & Wendel 2005; Paterson *et al.* 2006; Sémon & Wolfe 2007; Leitch & Leitch 2008; Tang *et al.* 2008; Anssour *et al.* 2009; Hardion *et al.* 2015). Currently, these topics are being updated in different plant families. Recent studies in Asteraceae, suggest that polyploidy has punctuated its evolutionary history and promoted its divergence from its sister clade Calyceraceae (Panero & Crozier 2016; Barker *et al.* 2016). Despite being an important phenomenon in the origin and diversification of composites and considering the great amount of species, there are very few studies concerning the implications and scopes of polyploidy in the different tribes.

The Vernonieae is one of the largest tribes of the Asteraceae family with around 1,600 species distributed in tropical regions of America, Asia and Africa (Keeley *et al.* 2007; Keeley & Robinson 2009). It is a very complex and variable group from biological and taxonomic points of view, known as "the evil tribe". Based on phylogenetic, morphological and molecular studies, the current classification recognizes 21 subtribes, of which 15 are from the New World and six from the Old World (Keeley & Robinson 2009). The subtribe Vernoniinae is the largest group of Vernonieae, with about 25 genera and 450 species, occurring mainly in South America. In this subtribe are comprised the species previously included in the *Lepidaploa* (Cass.) DC. section of the genus *Vernonia* Schreb., which have been mostly segregated into different genera (Bremer 1994; Robinson 1999; Keeley *et al.* 2007).

New World *Lessingianthus* H. Rob is one of the recently segregated genera, comprising 133 species distributed in Tropical South America (Robinson 1999; Dematteis 2006; Angulo & Dematteis 2015), and in Argentina there are 19 reported species (Angulo & Dematteis 2014). Angulo & Dematteis (2012) reported a considerable amount of polyploid taxa in this genus (67 % of a total of 42 analyzed taxa). For this reason, it is considered cytogenetically complex when compared to other genera of the Vernonieae tribe. Chromosome base number $x = 16$ is constant within the genus and somatic chromosome numbers ranged from 2n = 32 to 2n = 176 (Galiano & Hunziker 1987; Jones 1979; Ruas *et al.* 1991; Dematteis 1996, 1997, 1998, 2002; Dematteis & Fernández 2000; Dematteis *et al.* 2007; Oliveira *et al.* 2007; Angulo & Dematteis 2009, 2012, 2015). Angulo & Dematteis (2012) reported several cytotypes (diploids, tetraploids, hexaploids, octoploids, decaploid and one-decaploid) and species with mixed populations (diploids and tetraploids). These studies suggest an important role played by polyploidy in speciation and diversification within *Lessingianthus*. In spite of the high frequency of polyploidy in the genus, their polyploid origin (allo- or autopolyploidy) and fertility remains unknown.

Genome duplication affects the viability of reduced gametes (Otto & Whitton 2000; Ramsey 2007). The chromosomes of polyploid specimens are involved in multivalent associations during meiosis I, and exhibit irregular segregation in anaphase I, leading to abnormal gametes with unbalanced gene dosage (Stebbins 1940; Jackson 1976). In order to subsist in nature, polyploid species need to overcome these abnormalities. Commonly, polyploid species survive in nature by developing asexual modes of reproduction (Otto & Whitton 2000) such as vegetative multiplication (stolon, rhizome, tuber, bulb, among others) or apomixis (Batygina 2005). Asexual reproduction via apomixis produces seeds without paternal contribution and genetically identical to the mother plant, although there are some reports of genetic diversity among apomictic offspring (Baarlen *et al.* 2002; Ramage *et al.* 2004; Dias *et al.* 2018). According to Dijk & Vijverberg (2005) and Hand *et al.* (2015), asexual seed production came up independently from sexual ancestors, and currently there are 400 plant species representing 40 apomictic plant families (Carman 1997; Hörandl *et al.* 2008; Hojsgaard *et al.* 2014). In the great family Asteraceae, apomixis is well documented in 22 genera (Noyes 2007). Tucker & Koltunow (2009) and Whitton *et al.* (2008) strongly correlate apomixis to polyploidy, mainly to tetraploids. However, reproduction mechanisms

on *Lessingianthus* species are so far unknown; there are scarce and fragmented data on embryology and reproduction in Asteraceae that are mainly focused in species of economic value. Data about the reproduction mechanism might lead to a better understanding on the origin and persistence of the genus.

Lessingianthus plantaginoides (*Less.*) H. Rob. is a perennial tetraploid shrub with $2n = 4x = 64$ (Angulo & Dematteis 2012, 2014) that possess a disjunct distribution around the South of Brazil and the Northeast of Argentina and Uruguay. The studied population is located at a rocky outcrop named "Paraje Tres Cerros" that dates from the upper Jurassic to early Cretaceous (Herbst & Santa Cruz 1999). This rocky outcrop belongs to the Botucatu Stratigraphic Formation (Aceñolaza 2007) and includes three hills with animal and plant endemisms and high local biodiversity: Chico Mount (148 metres above mean sea level), Capará Mount (158 m.a.s.l.) and Nazareno Mount (179 m.a.s.l.). For all the above mentioned and taking into account not only the extensive distribution of this species around the hills, but also its high relative abundance that covered an extensive part of the region reaching up a height of 179 masl., and considering the absence of cyto-embryological data, the aims of this study were: (a) to perform an ontogenetic study of the male and female gametophytes of *L. plantaginoides*, (b) to carry out detailed meiotic analysis and evaluate the stainability of pollen grain, (c) to document and provide highlights on taxonomic implications of their reproductive aspects.

Materials and Methods

Plant material

Given the extensive distribution of the species, we collected up to five samples of three different populations of *L. plantaginoides* in the private natural reserve "Paraje Tres Cerros" (Argentina); Capará Mount (Population1, P1), Nazareno Mount (Population 2, P2) and Chico Mount (Population 3, P3) (Tab. 1). Figure 1 illustrates the habitat of the species.

The voucher specimens were deposited at the herbarium of the Instituto de Botánica del Nordeste (CTES). The sources of the samples are summarized in Table 1.

Embryological analysis

We fixed a total of ten floral buds and open flowers per populations in FAA (70% alcohol, formalin, acetic acid, 90:5:5). Permanent slides were obtained for the ontogenetic study. The material was dehydrated in a series of tertiary butyric alcohol and included in paraffin according to Johansen (1940). Transverse serial sections of 10–12 µm thick were cut with a rotary microtome. The sections were stained with Safranin-Astra blue (Luque *et al.* 1996) and then mounted with synthetic Canada balsam. The slides were examined under Leica DM LB2 (Leica, Wetzlar, Germany) light microscope equipped with a digital camera.

Meiotic analysis

A total of ten young flower buds per populations P2 and P3 (Tab. 1) were fixed in 1:5 lactic acid-ethanol (Fernández 1973) and stored in 70 % ethanol at 4 °C. Young anthers with microspore mother cells were squashed and stained with 2 % lactopropionic orcein (Dyer 1963). Slides were examined and photographed using a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) with digital camera Canon Power Shot A 640 (Tokyo, Japan). Meiotic behavior was analyzed in microspore mother cells at early prophase I, metaphase I and II (MI, MII), anaphase I and II (AI, AII), telophase I and II (TI, TII) and

Table 1 – Procedence of *Lessingianthus plantaginoides* populations.

Populations	Embyological studies	Meiotic studies
P1. Argentina. Corrientes. Dept. San Martín. Paraje Tres Cerros, Est. Higuera Cué, Cerro Capará. Pérez 29 (CTES)	+	
P2. Argentina. Dept. San Martín. Tres Cerros, Reserva Privada Tres Cerros, Cerro Nazareno. Pérez 31 (CTES)	+	+
P3. Argentina. Corrientes. Dept. San Martín. Paraje Tres Cerros, Reserva Privada Tres Cerros, Cerro Chico. Pérez 33 (CTES)	+	+

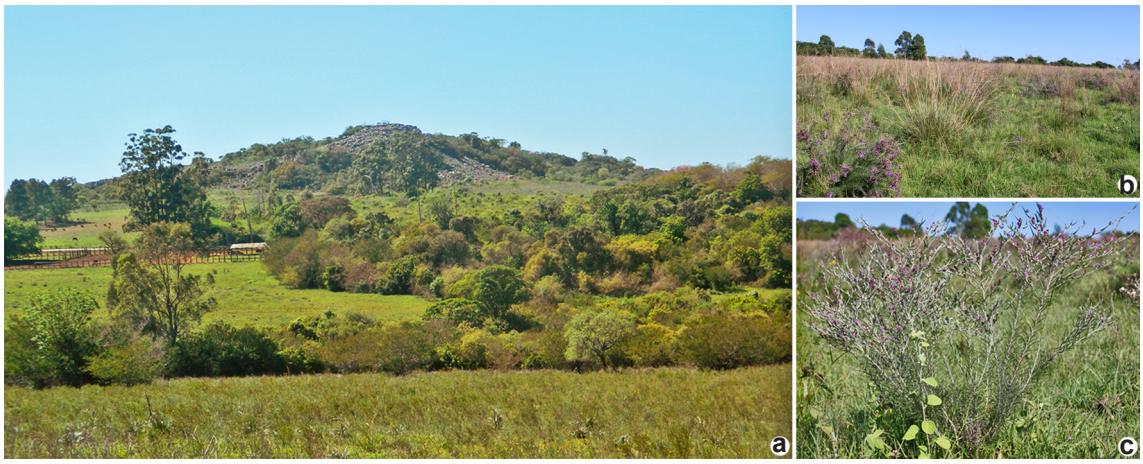

Figure 1 – a-c. Private Natural Reserve "Paraje Tres Cerros" (Argentina) – a. Chico Mount; b. population of *Lessingianthus plantaginoides* of the grassland; c. *L. plantaginoides* in the field.

tetrad spore stage. Analyses included description of chromosome pairing at prophase I, meiotic behavior at different stages and tetrads formation. Tetrads with four equal-sized microspores were considered normal. Simultaneously, we calculated the meiotic index (percentage of normal spores/ total spores analyzed per sample × 100).

To study the chromosome pairing, we observed a total of 20 microspore mother cells per population at prophase I.

Pollen stainability

We estimated pollen stainability with carmine: glycerine (1:1) staining (Pittenger & Frolik 1951). Approximately 500–1,000 pollen grains were analyzed per population sampled. Pollen stainability was calculated as percentage of stained pollen grains/total pollen grains per population analyzed.

Results

Androecium morphology

Androecium consisted of five elongated stamens, with its filaments fixed at the basal portion of the corolla throat. Each stamen comprised an anther and a filament; the anthers were sessile and linear, and had two thecae with two sporangia each (Fig. 2a). By the time of floral anthesis, the anther presented longitudinal dehiscence.

Microsporangium

Early in the ontogeny, staminal primordium consisted of homogeneous meristematic cells arranged in outer layer L1, medium layer L2 and innermost layer L3 (Fig. 2b). Meristematic cells were typically isodiametrical, thin-walled, with dense cytoplasm and a conspicuous nucleus. Anticlinal divisions occurred in meristematic layer L1 giving rise to the epidermal layer. Meanwhile, meristematic subepidermal layer L2 increased the mitotic divisions towards the central region of the primordium. These mitotic divisions gave rise to a tetralobulate anther (Fig. 2c-e). Further mitotic divisions on L2 originated an archesporial row of cells (Fig. 2c). The archesporial cell divided periclinally and developed two different cell lines: we observed in Fig. 2d the primary sporogenous cell and the primary parietal cell. The primary sporogenous tissue directly becomed the sporogenous tissue (Fig. 2e). Periclinal divisions on primary parietal cells originated two layers: an internal secondary parietal layer and an external secondary parietal layer (Fig. 2d,e). The internal secondary parietal layer (Fig. 2e) gave rise to the tapetum (Fig. 2f,f'), meanwhile the external secondary parietal layer (Fig. 2e) formed the endothecium and a middle layer (Fig. 2f,f') after the periclinal division.

The central region of the anther primordium, occupied by the innermost layer L3, increased in size and volume (Fig. 2b-e). The procambium developed from L3, and later resulted in the central vascular bundle of the anther. The connective tissue and septum between the two pollen sacs also originated from L3 (Fig. 2b-d,f).

The young anther is tetrasporangiate in transverse section (Fig. 2a,c-e). Mature anther wall

consisted of epidermis, middle layer, endothecium and tapetum (Fig 2f). The epidermis persisted until tetrad stage, as rectangular uninuclear cells (Fig. 2f-h). During anther dehiscence, the epidermal cells crushed (Fig. 2n). The middle layer was ephemeral and consisted of flattened rectangular cells observed during microspore mother cell stage (Fig. 2f). Because the middle layer shared a common origin with the endothecium, the anther wall development is of Dicotyledonous type (Fig. 2f'). Finally, the middle layer is compressed and obliterated before the microsporangium achieved maturity (Fig. 2g). In young anthers the endothecium consisted of elongated cells with

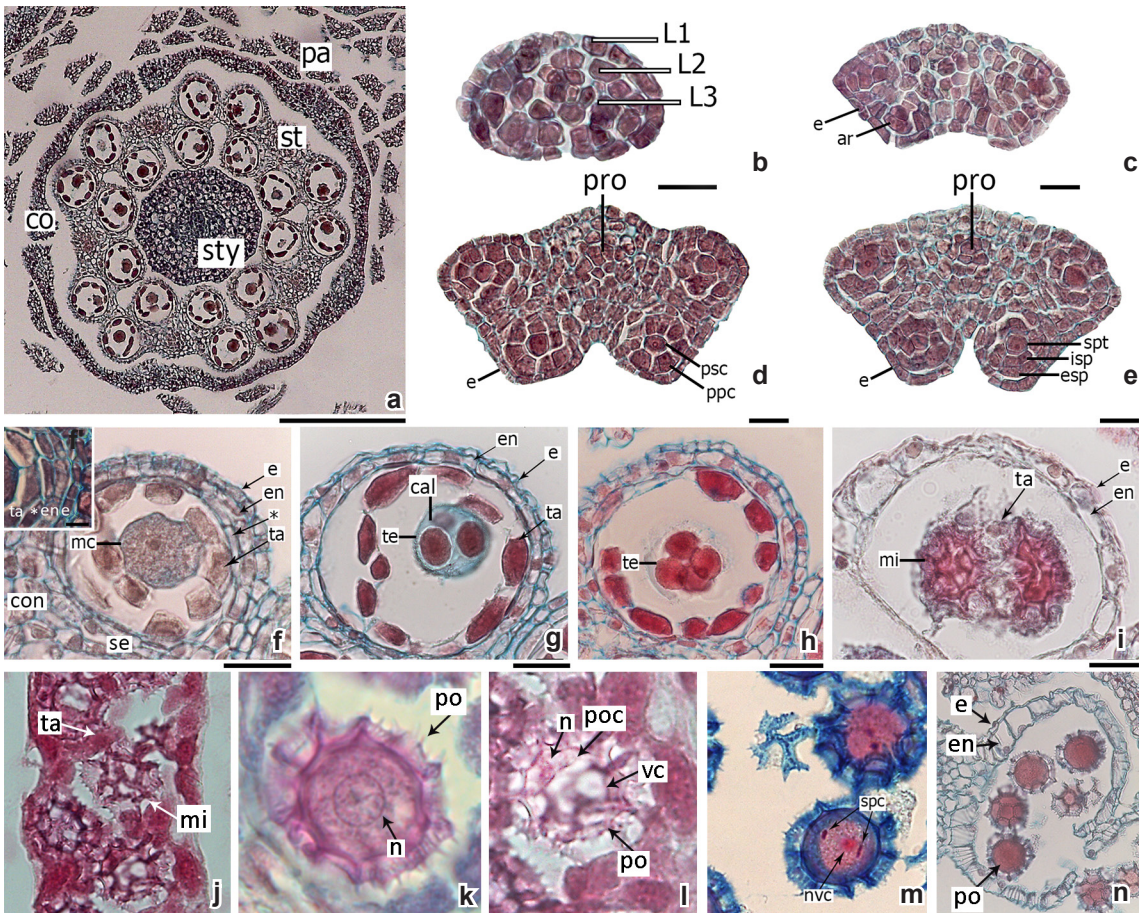

Figure 2 – a-n. Male gametophyte development of *Lessingianthus plantaginoides* – a. cross section of a flower; b. cross section of anther primordia; c. cross section of young anther with archesporial cell; d. young anther with primary parietal cell and primary sporogenous cell; e. young anther showing sporogenous tissue, internal and external secondary parietal layer; f. anther locule containing the microspore mother cell; f'. detail of a young anther wall; g. anther locule showing a tetrad with callose wall; h. anther locule with a tetrahedral tetrad with slight callose wall; i. anther locule with a young microspore surrounded by tapetal cells; j. longitudinal section of anther locule showing the tapetal cells around the young microspores; k. young pollen grain unicelled; l. young pollen grain unicelled with vacuole; m. mature pollen grain three-celled; n. mature anther in anthesis. (*ar* = archesporial cell; *cal* = callose; *co* = corolla; *con* = connective tissue; *e* = epidermis; *en* = endothecium; *esp* = external secondary parietal layer; *isp* = internal secondary parietal layer; *L1* = primary layer; *L2* = secondary layer; *L3* = tertiary layer; *mc* = microspore mother cell; *mi* = microspore; *n* = nucleus; n*vc* = nucleus of the vegetative cell; *pa* = papus; *po* = pollen grain; *poc* = pollen grain cell; *ppc* = primary parietal cell; *pro* = procambium; *psc* = primary sporogenous cell; *se* = septum; *spc* = sperm cell; *spt* = sporogenous tissue; *st* = stamen; *sty* = style; *ta* = tapetum; *te* = tetrads; *vc* = vacuole; * = middle layer). Scale bars: a = 200 µm; k = 50 µm; b, c, d, e, f, g, h, i, j, l = 20 µm.

prominent nuclei (Fig. 2f-h) forming a continuous layer of rectangular cells that were larger than the epidermal cells. During the maturation of anther, the tangential and radial walls of the endothecium acquired lignified thickenings (Fig. 2i,n). The tapetum was of secretory type, consisted of rectangular cells, of dense cytoplasm indicating high metabolic activity; this tissue behaved differently during development. At first, the cells persisted *in situ*, and displayed high metabolic activity (Fig. 2f,g). Further in development, the tapetal cells started to protrude the anther locule. Consecutively, at free microspore stage, the tapetal cells completely invaded the locule and surrounded the young microspores (Fig. 2i,j). Finally, at pollen grain stage the tapetal cells degenerated completely (Fig. 2n).

Microsporogenesis

The sporogenous tissue consisted of a single vertical row of isodiametrical cells with prominent nuclei (Fig. 2e). These cells developed into microspore mother cells, which were voluminous, spherical, with conspicuous nuclei and dense cytoplasm (Fig. 2f). This cells entered Meiosis and formed tetrahedral tetrads, covered of callose (Fig. 2g). Cytokinesis was of the simultaneous type. Later in development, the callose wall disintegrated (Fig. 2h) and the tetrads released four microspores with a thin immature exine wall.

Microgametogenesis

The released microspores free in the anther locule (Fig. 2i-k) were richly cytoplasmic with a prominent and centrally placed nucleus (Fig. 2k). The microspores increased in volume and presented a centrally placed large vacuole (Fig. 2l); they underwent a mitotic division, resulting in a large vegetative cell and a small generative cell. The vegetative cell grew and enclosed the generative cell in its cytoplasm; soon after, the generative cell undergoes a second mitotic division and formed two sperm cells (Fig. 2m). Finally, the mature pollen grain was released at the tricellular stage (Fig. 2m,n).

Meiotic analysis

Two populations of *L. plantaginoides* were cytogenetically studied. The results presented correspond to populations of Chico and Nazareno Mount (Tab. 1). All microspore mother cells presented different chromosome meiotic configurations, including univalent (I), bivalent (II) and tetravalent (IV) associations. The most frequently configuration in eight of observed cells was 6 I+27 II+1 IV (Fig. 3a).

The two populations of *L. plantaginoides* presented similar rate of chromosome regularity, but some abnormalities were registered. The analysis of a total of 746 microspore mother cells at metaphase I-telofase II revealed 97.72 % of normality. Only 2.27 % of the observed cells demonstrated irregular behavior. Meiotic irregularities were only observed at the earliest phases (MI and AI) of the first meiotic division, while second meiotic division was completely normal. Results of each analyzed phase are detailed in Table 2.

During the first metaphase, P2 and P3 had respectively 97.7 % and 98.2 % of cells with normal behavior of the chromosomes. The remaining cells presented off-plate chromosomes (Fig. 3b). Notwithstanding, cells observed at metaphase II were normal. At anaphase I, the rate of regular microspore mother cells observed was similar, with 93 % in P2 and 94 % in P3. The remaining observed cells presented bridges (app. 3 %) (Fig. 3c) and bridges with fragments (app. 2 %) (Fig. 3d) in both populations. The anaphase of the second division was normal.

Meiotic index was high in both populations, reaching 98 %. However, abnormal sporads were observed in low proportion (Tab. 3). These include 0.44 % of triads, 0.89 % of dyads and 0.44 % of monads in P2; and 0.63 % of triads, 0.75 % of dyads and 0.50 % of monads in P3.

Pollen stainability

Pollen stainability resulted to be relatively high in both populations sampled with 97 % of stained pollen grains (Fig. 3e). Nevertheless, the remaining pollen grain showed abnormal size and no cytoplasm. Empty pollen grains were relatively few, P2 with 0.86 % and P3 with 0.53 %. Furthermore, pollen grain with abnormal size was slightly higher in P3 (2.13 %) than in P2 (1.67 %) (Fig. 3f). Table 4 summarized the obtained percentages.

General characteristics of the ovary

The ovary was inferior, bicarpellary, syncarpous and unilocular. Inside, it housed a single basal ovule which resulted to be anatropous, unitegmic and tenuinucellate (Fig. 4a).

The ovule primordia arose from the floor of the ovary and grew vertically (Fig. 4b). By then, the

body of the ovule is composed by three cell layers: dermal (L1), subdermal (L2) and central (L3) (Fig. 4b). The three layers underwent successive mitotic divisions, so that the ovule primordia get inverted and curved reaching the anatropous disposition (Fig. 4c-e).

Megasporogenesis

The initial archesporial cell, distinguished by its larger volume, dense cytoplasm and prominent nucleus, differentiated in the subdermal layer (L2) of the ovule (Fig. 4e,f). This cell enlarged and directly functions as the megaspore mother cell (Fig. 4g). Rarely, approx. in 2 % of the observed cases, the megaspore mother cell was accompanied by a developed and differentiated nucellar cell that showed high metabolic activity (Fig. 4h). The megaspore mother cell underwent two meiotic divisions (meiosis I and II), producing first a dyad, and then a linear shaped tetrad of haploid megaspores. The chalazal megaspore grew faster, it enlarged and crushed the other three micropilar megaspores of the tetrad (Fig. 4i). The chalazal megaspore became the functional megaspore that develops further into a megagametophyte.

Megagametogenesis

The reduced megaspore at the chalazal end increased in volume and size. Simultaneously, the inner epidermis of the integument differentiated into the endothelium, a layer of radially elongated cells, with dense cytoplasm and prominent nuclei (Fig. 4m-p). The endothelium remained single-layered throughout the megagametophyte development.

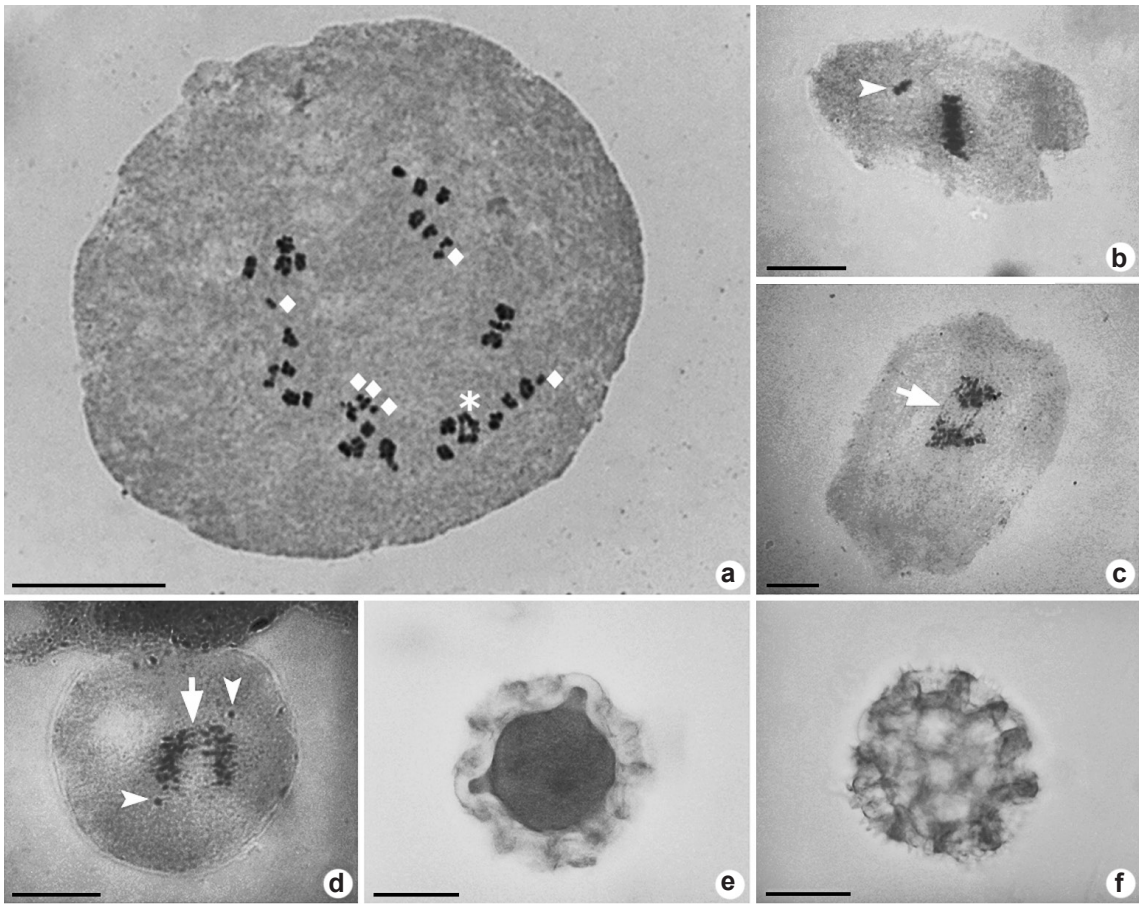

Figure 3 – a-f. Meiotic behavior of *Lessingianthus plantaginoides* – a. chromosome configuration at diakinesis-metaphase I with 6 I, 26 II, 1 IV; b. metaphase I with chromosome outside plate; c. anaphase I with bridges; d. anaphase I with bridges (narrow) and fragments (narrowheads); e. fertile pollen grain; f. sterile pollen grain. (*rhombus* = univalent chromosomes; *asterisk* = tetravalent association; *arrowheads* = chromosomes outside plate; *arrows* = bridges in metaphase I). *Scale bars*: a = 20 µm; b, c, d, e, f, g, h, i = 10 µm.

Table 2 – Percentage of meiotic abnormalities founded in Population 2 (P2) and 3 (P3).

	P2	**P3**
metaphase I	%	%
Regular	97.7	98.2
Chromosomes outside plates	2.25	1.8
Total of cells analyzed	133	112
anaphase I	%	%
Regular	94.3	93.3
Bridges with fragment	2.24	2.83
Bridges without fragment	3.37	3.77
Total of cells analyzed	89	106

Late in development, the chalazal megaspore underwent three cycles of mitotic divisions, giving rise to two nucleate (Fig. 4j), then four nucleate (Fig. 4k,l) and finally, eight nucleate embryo sac. Thus, an eighth-celled coenocytic megagametophyte is formed.

The eight previously formed nuclei are reorganized to form a mature embryo sac (Fig. 4m-r). The organized female gametophyte consisted of an egg apparatus, the central cell, and antipodal cells (Fig. 4q). At the chalazal side of the mature embryo sac, two or three small antipodal cells were observed (Fig. 4q). At the micropylar side, the egg apparatus consisted of a well-developed egg cell and two elongated synergids (Fig. 4m-o). The extreme micropylar pole of the synergids contained a conspicuous filiform apparatus, a structure with a strongly thickened wall. The central cell with two polar nuclei and several vacuoles are centrally placed and occupied the largest portion of the embryo sac (Fig 4 m). Tetraploid *L. plantaginoides* had monosporic type of megasporogenesis and the megagametophyte followed a Polygonum type development.

Discussion

This is the first male and female ontogenetic description of the genus *Lessingianthus*, until date data about embryology was restricted to seed and pollen morphology. Additionally, there were no studies that linked polyploidy to meiotic behavior and the mode of reproduction of the analyzed species. Moreover, this is the first record on the male and female gametophyte development of a South American Vernonieae.

Male gametophyte development, meiotic behavior and pollen viability

The male data provided here agrees with various characteristics of other members previously reported in the tribe. Features of taxonomic importance cited by Herr (1984) such as number of microsporangia, type of wall development, number of layers, persistence of the epidermis, form of tetrads and number and shape of pollen grains at shedding stage, were consistent within tribe Vernonieae (Pullaiah 1979). Following Robinson's classification (1999), Vernonieae Tribe

Table 3 – Post-meiotic products and meiotic index (MI) in *Lessingianthus plantaginoides*.

P	**Sporads resulting of meiosis**			**Total of analyzed sporads**	**MI %**
	Monads %	**Dyads %**	**Triads %**		
P2	0.44	0.89	0.44	890	98.2
P3	0.50	0.75	0.63	791	98.1

P = population; MI = Meiotic Index

Table 4 – Percentages of pollen grain type and pollen stainability in *Lessingianthus plantaginoides.*

P	Pollen grains analyzed	Normal	Abnormal		Pollen viability
			no cytoplasm (%)	small size (%)	
P2	1267	1235	0.86	1.67	97.47 %
P3	748	728	0.53	2.13	97.32 %

is subdivided into 11 subtribes, of which only *Elephantopodinae* and *Vernoniinae* were studied from an embryological point of view (Pandey & Singh 1980).

In *L. plantaginoides*, the primary sporogenous cells directly acted as microspore mother cells and the sporogenous tissue displayed a single row of microspore mother cells. This particular feature is the consequence of transversal divisions on the primary sporogenous cells and was also reported in *Elephantopus scaber L.*, *Vernonia elaeagnifolia* D. and *V. divergens* Benth., members of *Elephantopodinae* Less. and *Vernoniinae* Lessing. subtribe respectively. This is not a common feature among flowering plants and its full biological significance on plant phylogeny remains unclear (Pan *et al.* 1997). Until date, embryological data on the remaining genera of the subtribe is still unreported. The traits described in this study will be useful to clarify taxonomic relationships and to compare with other related groups.

As many authors agree, polyploidy constitutes a key feature in plant evolution (Grant 1989; Soltis & Soltis 1993, 1999; Ramsey & Schemske 2002). In *Vernonieae* tribe, this phenomenon is usual, and among the new world genera, *Lessingianthus* is the genus with the major proportion of polyploid species and the highest ploidy levels known (Angulo & Dematteis 2012). Previous studies as Galiano & Hunziker (1987), Jones (1979), Ruas *et al.* (1991), Dematteis (1996, 1997, 1998, 2002), Dematteis & Fernández (2000), Oliveira *et al.* (2007), Dematteis *et al.* (2007) and Angulo & Dematteis (2009, 2012) have been dedicated to report chromosome numbers of this entities, but none of them revealed the meiotic behavior. This study represents the first report of meiotic behavior of a polyploid entity of this genus.

Meiotic stability is one of the main hurdles that newly formed polyploid have to face (Baduel *et al.* 2018). Hollister *et al.* (2012) and Yant *et al.* (2013) also emphasize that there are genes controlling meiotic recombination and crossover that are strongly implicated in polyploid adaptation. In autopolyploid species of recent origin, the genetic modifications following a chromosomal duplication event needed in order to stabilize chromosome pairing and migration during division, have not yet been carried out, therefore they form numerous multivalent of abnormal segregation (Ramsey & Schemske 2002; Adams & Wendel 2005; Comai 2005; Bomblies *et al.* 2015). *L. plantaginoides* formed a high percentage of bivalents and few mono and tetravalents during meiosis. The presence of tetravalent associations suggests that this species is autopolyploid, and this idea is supported by karyotype analysis performed previously, where chromosomal groupings were found (Angulo & Dematteis 2015). As in other polyploid species, *e.g.*, *Arabidopsis arenosa* (L.) Lawalrée (Brassicaceae) (Yant *et al.* 2013), in *L. plantaginoides* also the meiotic instability is overcome, becoming an established autopolyploid with sexual reproduction.

According to Baduel *et al.* (2018), two of the long terms gains of polyploid species are the potential colonization and enhanced invasiveness. Currently, there is an increasing number of scientists who subscribe to the association between polyploidy, the potential for habitat colonization and transitions to weedness (Soltis P & Soltis 2000; Prentis *et al.* 2008; Pandit *et al.* 2011; Beest *et al.* 2012). In this sense, an established autopolyploid has already solved the genetic and mechanistic aspects concerning chromosome miss-segregation and altered gene expression (Baduel *et al.* 2018) that limit the expansion of the new cytotype. Once these limitations have been overcome, the colonization seems to be enhanced. Particularly in *L. plantaginoides,* this is clearly demonstrated given the wide expansion of the population all over the hills.

On the other hand, it is expected that species with genome doubling present high percentage of abnormalities and generates high rates of sterile

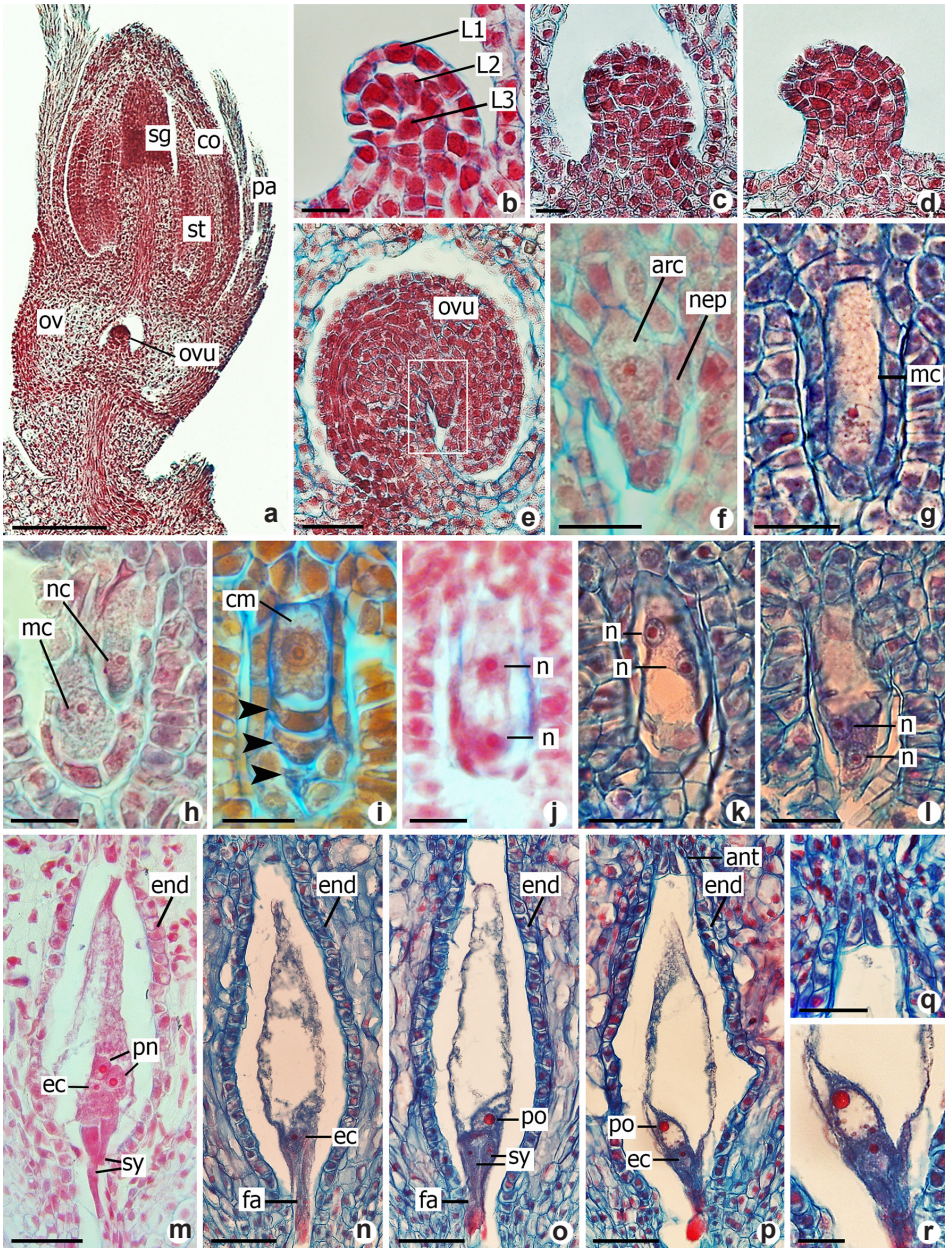

Figure 4 – a-r. Female gametophyte development of *Lessingianthus plantaginoides* – a. longitudinal section of a flower; b. longitudinal section of the ovule primordium; c. ovule primordium enlarged; d. ovule primordium curved; e. solitary anatropous ovule completely curved; f. detail of the archesporial cell; g. enlarged megaspore mother cell; h. megaspore mother cell accompanied by an extra cell; i. chalazal megaspore developed and micropile megaspores crushed (*arrowheads*); j. two-nucleate embryo sac; k-l. four-nucleate embryo sac – k. two nucleus with chalazal orientation; l. two nucleus with micropile orientation; m. embryo sac with two polar nuclei; n-o. mature embryo sac – n. egg cell and part of the filiform apparatus; o. female gametophyte with two synergids; p-r. female gametophyte with starch grains – p. female gametophyte with nucleus formed by fusion of polar nuclei and egg cell; q. detail of the antipodals; r. detail of the polar nuclei with starch grains and the egg cell behind. (*ant* = antipodals; arc = archesporial cell; *co* = corolla; *ec* = egg cell; *end* = endothelium; *fa* = filiform apparatus; *L1* = primary layer; *L2* = secondary layer; *L3* = tertiary layer; *mc* = megaspore cell; *n* = nucleus in female gametophyte; *nc* = nucellar cell; *nep* = nucellar epidermis; *ov* = ovary; *ovu* = ovule; *pa* = papus; *pn* = polar nuclei; *po* = nucleus formed by fusion of two polar nuclei; *sg* = stigma; *st* = stamen; *sy* = synergids). Scale bars: a = 200 µm; e, f, m, n, o, p = 50 µm; b, c, d, g, h, i, j, k, l, q, r = 20 µm.

pollen (Ramsey & Schemske 1998). Instead of that, the meiotic behavior resulted to be normal, and this correlates with the meiotic index obtained from sporads and a high stainability of pollen grains. *L. plantaginoides* only presented low rates of abnormalities during metaphase I and anaphase I. Throughout metaphase I, some chromosomes showed precocious migration and were visible as out of plate chromosomes. Ortiz *et al.* (2011) observed the same in tetraploid species of *Arachis* sect. *Rhizomatosae* Krapov. & W.C. Greg. and related it to the presence of only one terminal chiasmata per bivalent. This abnormality appears to be common among polyploid specimens; was also reported in related genera such as natural populations of *Campuloclinium macrocephalum* DC. (Eupatorieae) (Farco & Dematteis 2014), and in six species of *Crhysolaena* (H. Rob.) (Vernonieae) (Via Do Pico & Dematteis 2012). Seijo & Solís Neffa (2006) correlated the presence of out of plate chromosomes with the formation of additional micromicrospores (spores with abnormal reduced size). Nevertheless, in this case the sporads did not show micromicrospores, and only a small percentage of cells was affected, thus this abnormality did not seriously affect the gamete formation. The high meiotic stability and the number of bivalents indicates that *L. plantaginoides* is an autopolyploid with a highly diplodized complement. Further analysis of other members of the genus with different ploidy levels should greatly improve our understanding of their evolution and therefore the processes of polyploid speciation in the tribe.

Sexuality, ploidy level and female gametophyte development

The following female embryological traits of *L. plantaginoides* correspond to those of Asteraceae family (Davis 1966; Johri *et al.* 1992): anatropous, unitegmic and tenuinucellate ovules with a single hypodermal archesporial cell, which directly undergo megasporocyte with well-developed endothelium. In *Lessingianthus* genus, only the morphology, anatomy and ontogeny of the pericarp and pappus of *L. brevifolius* (Less.) H. Rob. were described (Martins & Oliveira 2007). The description of the ovary of *L. plantaginoides* given in this study matches the previously mentioned description of *L. brevifolius* (Martins & Oliveira 2007). Both species have inferior, bicarpellate, syncarpous and unilocular ovary with a single basal ovule attached to it. These features also match with the description of other members of the tribe Vernonieae such as *Vernonia elaeagnifolia* DC., *V. divergens* (DC.) Edgew., *Elephantopus scaber* L., *Adenostemma rugosum* Wt. and *A. lavenia* (L.) Kuntze from Indian district (Pullaiah 1979). Nevertheless, Pullaiah (1979) reported that *E. scaber* presented two ovules per ovary in 3% of the analyzed ovaries. These ovules were arranged face to face, shared a common funicle and the gametophytes developed synchronously and showed normal features. *L. plantaginoides* did not show this feature, however more studies on other subtribes are necessary to understand the extent of this particular feature.

Lessingianthus plantaginoides developed a monosporic embryo sac of the Polygonum type. Because the female gametophyte develops from a single meiotically reduced megaspore, it is a monosporic embryo sac. This embryo sac development is of Polygonum type due to the chalazal orientation of the developed megaspore, the number and arrangement of the nucleus in the mature embryo sac (Willemse & van Went 1984; Pandey 1997; Lersten 2004). Almost 70% of angiosperms share the Polygonum type of development (Reiser & Fischer 1993) is highly frequent among flowering plants and there are several number of cytological configurations leading to it (Willemse & van Went 1984). Nevertheless, Asteraceae shows great variability on gametophyte development: bisporic development (Bergman 1942; Harling 1951), Polygonum and Allium type (Tiagi & Taimni 1960), Pyrethurum parthenifolium type (Ervandyan 1975), Fritillaria type (Maheshwary & Srinivasan 1944), Drusa type (Liu 2001) and Oenothera type (Teng *et al.* 2008; Li *et al.* 2009). This family also shows great variability on asexual reproduction. Apomixis is a common type of asexual reproduction among Asteraceae (Noyes 2007). We were not able to find any mature apomictic embryo sacs in *L. plantaginoides*, nevertheless we found some cells that differentiated from the nucellus and accompanied the megaspore mother cell. It seems possible that some cells around the megaspore mother cell entered in activity, but none of them comes to form a mature asexual embryo sac. Attempting to explain this result we are tempted to think that the persistence in low frequency of a nucellar cell that eventually enter in activity might be a non-functional or relictual condition of an ancestor with apomictic reproduction. Instead of that, other authors suggests that apomictic

phenomena are controlled by environmental factors such as light, temperature regimen and the choice of pollinator (in facultative apomicts) (Asker 1980; Koltunow 1993), so the development of a nucelar cell might be a random event related to the environmental factors mentioned above. In any case, more studies in other *Lessingianthus* species are required in order to clarify it.

Sexual reproduction is valuable for plant species since it can provide a high percentage of genetic diversity, and it may help to buffer deleterious mutations (Rice 2002; Otto & Gerstein 2006). This mode of reproduction is highly costly in terms of energy investment (Jönsson 2000). A polyploid species had to face different environmental obstacles and ecological challenges, and also take into account the ecological competition with their diploid counterparts (Otto & Whitton 2000). In this sense, the mode of reproduction plays a vital role in the establishment of the new cytotype, and the embryological traits are investments that ensure progeny to the new generation. These features are usually consistent within genera and plant families, and combined with anatomical or molecular data have helped to clarify the relationships among angiosperms (Endress 2003; Hojsgaard *et al.* 2008; Oriani & Scatena 2014). Female embryological traits as ovary architecture, ovule shape and number of teguments, are considered of evolutionary and taxonomic importance by Herr (1984), Tobe (1989), Pandey (1997) and Endress (2011). This study provided the first embryological data of the genus, nevertheless other *Lessingianthus* members have to be study in order to reach a broader comprehension of the role of polyploidy in the diversification of the genus, and to determine the embryological traits of evolutionary significance.

This work strongly indicates that *L. plantaginoides* reproduces sexually and this finding reveals that ploidy level does not affect its fertility. In the light of the results this species is a well-established paleotetraploid. Ancient polyploidy is common in the family (Barker *et al.* 2008), Barker *et al.* (2016) demonstrated that the family shares with Calyceraceae a paleotetraploid ancestor and the more derived Asteraceae tribes are descendants of a paleohexaploid. The different chromosome base numbers and the presence of polyploid species with different ploidy levels suggest a complex history of polyploid speciation in the tribe; research of this topic in other less studied genus are necessary to a better understanding.

This is the first cyto-embriological description for a South American Vernonieae. The male gametophyte presented Dicotyledonous type of anther wall development, four-celled anther wall, tetrahedral tetrads and mature 3-celled pollen grains. Despite its ploidy level, *L. plantaginoides* had normal meiotic behavior; the sporads were tetrads of equal size and presented a high percentage of pollen stained. The female gametophyte presented a single anatropous ovule, with one tegument; the embryo sac followed a monosporic Polygonum type of development. No mature apomictic embryo sacs were found. The traits described in this study would be helpful for future phylogenetic studies. The results indicate that *L. plantaginoides* is a highly diplodized autotetraploid with sexual reproduction. The abundance of the species in the hills demonstrates the colonizing potential it has achieved.

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