# A pentaploid endosperm and a Penaea-type embryo sac are likely synapomorphies of Azorella (Apiaceae, Azorelloideae)

**Jan Ptáček**
Charles University
**Petr Sklenář**
Charles University
**Jan Pinc**
Charles University
**Romana Urfusová**
Charles University
**Carolina I. Calviño**
Instituto de Investigaciones en Biodiversidad y Medioambiente (INIBIOMA), CONICET – Universidad del Comahue
**Tomáš Urfus** ( tomas.urfus@natur.cuni.cz )
Charles University

**Research Article**

**Keywords:** Andes, Azorelloideae, flow cytometry, Penaea-type embryo sac, pentaploid endosperm.

**Posted Date:** May 19th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1645824/v1

# Abstract

About 80% of angiosperms form a monosporic *Polygonum*-type embryo sac whereas in the remaining species eleven other types of embryo sac are found. Evidence as to the type of embryo sac is lacking for many plant species, and the role of higher-ploidy endosperm is unknown. In contrast to the rest of the Apiaceae, where a *Polygonum*-type embryo sac (3n endosperm) has been reported, the few species of the Azorelloideae studied form a *Drusa*-type embryo sac (3n endosperm) or a *Penaea*-type embryo sac (5n endosperm). This variation within Azorelloideae makes this subfamily, and its genus *Azorella* in particular, a good candidate for studying the evolutionary importance of the embryo sac and endosperm in diversification. We studied the variation in the type of embryo sac and the ploidy level of the endosperm in Andean-Patagonian *Azorella* and closely related *Pozoa* on a sample of 101 individuals from 31 populations of 21 species. We employed flow cytometric seed screening and calibrated the results of ploidy level estimation against embryological observations. In addition, we examined the genome size variation of the species sampled. All species of *Azorella* formed *Penaea*-type embryo sacs and a pentaploid endosperm whereas one species of *Pozoa* formed triploid and the other tetraploid endosperms. Variations in the type of embryo sac and endosperm ploidy have probably shaped the evolution of the different lineages of Azorelloideae in the southern Andes. A *Penaea*-type embryo sac, which represents a likely synapomorphy of *Azorella*, is a feature of underestimated significance in the evolution of angiosperms.


# Introduction

The Angiosperm Terrestrial Revolution is tightly linked to key evolutionary novelties in the reproductive and vegetative biology of flowering plants (Benton et al. 2022). Among the reproductive innovations, not only flowers, but also the embryo sac and double fertilization leading to the formation of nutritive endosperm have diversified to an extent unseen in any other lineage of land plants (Friedman 1998; Benton et al. 2022). Although plant reproductive patterns have been studied extensively (Ebert and Greilhuber 2005; Caparelli et al. 2006), little is known about the variation in embryo sac and endosperm formations within plant families (Rangan 2020).

Variation in the type of embryo sac across angiosperm lineages includes developmental and structural differences of the female gametophyte. These have resulted in the delimitation of numerous types of embryo sac (e.g. *Polygonum*-type, *Oenothera*-type, *Allium*-type, *Drusa*-type, *Penaea*-type, etc.; Maheshwari 1937; Friedman et al. 2008). These are recognized based on the number of megaspore nuclei that initiate the gametophyte (i.e. gametophyte of a monosporic, bisporic, or tetrasporic origin), on the final number of nuclei in the mature embryo sac (i.e. gametophyte 4, 8 or 16-nucleate), and on the final disposition and function of its cells (Maheshwari 1937; Friedman et al. 2008). This variation in angiosperm gametophytes ultimately creates variation in endosperm genetics and ploidy (e.g. the degree of heterozygosity, maternal to paternal genomic ratios, etc.), which is likely subject to selection and is therefore of evolutionary importance (Friedman et al. 2008).

Approximately 80% of angiosperm species in 239 families have a *Polygonum*-type embryo sac (Kordyum and Mosyakin 2020), i.e. monosporic, 8-nucleate and with a micropillar pole with a three-celled egg apparatus, a chalazal pole with three antipodals and a central cell with two nuclei, giving rise to a triploid endosperm upon fertilization. The prevailing hypothesis, based on phylogeny, is that the *Polygonum*-type embryo sac has evolved only once in angiosperm evolution and was the ancestral condition of crown angiosperms (Friedman et al. 2008; Kordyum and Mosyakin 2020; but see Williams and Friedman 2002) for alternative hypotheses).

Over more than a hundred years, embryological observations have resulted in description of different types of embryo sac. These have been named after particular families or genera where they were first observed, but were later found also in unrelated families (Maheshwari 1937). For example, the tetrasporic *Penaea*-type embryo sac was first discovered in the family Penaeaceae (Stephens 1909) and later found in the Apiaceae (Håkansson 1923, 1927), Euphorbiaceae (Subba Rao 1937) and Malphighiaceae (Fagerlind 1938). These independent origins of embryo sac types support the notion that the developmental and structural characteristics of the female gametophyte are highly homoplasious among the angiosperms and therefore potentially adaptive (Friedman et al. 2008). Although the evolutionary importance of the embryo sac and the type of endosperm seems unquestionable, information on their variation within families or genera is scarce.

In the Apiaceae, species of three of its four subfamilies (i.e. Apioideae, Saniculoideae and Mackinlayoideae) have been studied embryologically, and all of them have been found to have a *Polygonum*-type embryo sac and therefore a 3n endosperm, with no other embryo sac type reported for these subfamilies (Jurica 1922; Håkansson 1923, 1927, 1952; Tseng 1967; Henwood and Hart 2001). However, within the Azorelloideae, the few species studied display a tetrasporic, 16-nucleate, *Drusa*-type embryo sac and 3n endosperm (in *Bowlesia incana* and *Drusa oppositifolia*) or a tetrasporic, 16-nucleate, *Penaea*-type embryo sac and 5n endosperm (in *Azorella trifurcata* and *A. spinosa*; Håkansson 1923, 1927, 1952; Tseng 1967; Henwood and Hart 2001). This variation within the Azorelloideae makes this subfamily a good model group for studying the evolutionary importance of the embryo sac and endosperm in its diversification, and particularly within *Azorella*, the richest genus within the subfamily.

*Azorella* species are dominant cushion-forming plants in the Andes and in Patagonia. Moreover, their nursing effect on other high-elevation plant species makes them key in the maintenance of the biodiversity of these regions (Martinez 1989; Nuñez et al. 1999; Sklenář 2009; Calviño et al. 2016; Fernández et al. 2016, 2017a). Based on phylogeny, the genus is currently treated in a broad sense, that is, encompassing the former smaller genera *Huanaca* Cav., *Laretia* Gilliesh & Hook., *Mulinum* Pers., *Schizeilema* (Hook.f.) F. Muell. and *Stilbocarpa* (Hook.f.) A. Gray (Plunkett and Nicolas 2017; Fernández and Calviño 2019; Fernandez et al. 2020). *Azorella* sensu Fernández and Calviño (2019) comprises 58 species divided between two main monophyletic lineages formalized as subgenera: the Austral lineage (subgenus *Azorella*), which includes 23 species distributed in Austral regions of the Southern Hemisphere, including Patagonia, Australia, New Zealand and the subantarctic islands, and the Andean-Patagonian lineage (subgenus *Andinae*) comprising 35 species mainly of South American distribution in high-elevation regions of the Andes and Patagonia (Fernández and Calviño 2019). The genus is further divided into ten sections (corresponding to the main sub-lineages; Plunkett and Nicolas 2017) that represent monophyletic groups or species of uncertain position (Andersson et al. 2006; Nicolas and Plunkett 2009, 2012; Fernández et al. 2017a, b; Plunkett and Nicolas 2017; Fernández and Calviño 2019). Morphological synapomorphies support the monophyly and diversification of subgenus *Andinae*, specifically a woody cushion habit (Fernández et al. 2017a). However, no morphological synapomorphies have been described for the whole genus (Fernández et al. 2016, 2017c). Given the above-mentioned embryological observations within the Azorelloideae, a tetrasporic, 16-nucleate embryo sac is suspected as synapomorphic for the whole subfamily, although this has not been examined empirically for most of its genera, including its most speciose genus *Azorella*. This and the phylogenetic positions of the azorelloids (Nicolas and Plunkett 2009) studied embryologically so far led us to hypothesize that the *Penaea*-type and therefore a higher ploidy level (5n) of endosperm are synapomorphies of the genus *Azorella*.

In this study, we sampled nineteen species of *Azorella* and two species of *Pozoa.* This amounts to a five-fold increase in the number of species screened for embryo sac and endosperm variation within the Azorelloideae. We combined, for the first time, classical embryological techniques with quantitative flow cytometric seed screening to analyse the type of embryo sac and endosperm ploidy level in each species. The resulting data allowed us to test our aforesaid hypothesis regarding the evolution of the embryo sac and endosperm within the Azorelloideae.

## Materials And Methods

# Field sampling

Populations of *Azorella* and *Pozoa* were sampled during 2016–2022 in the temperate and tropical Andes of Argentina, Bolivia and Ecuador. Each sampling site was characterized by geographic coordinates, elevation and a general habitat description (see Supplementary Table 1 for locality details). In total, samples of 101 individuals from 31 populations (Fig. 1), corresponding to 19 species of *Azorella* and two species of *Pozoa* Lag. were collected (96 leaf samples for absolute genome size estimation, 276 fruit samples from 24 individuals for the determination of endosperm ploidy and 4 flower samples for embryology; see Table 1). This sampling of *Azorella* includes representatives from the two main lineages of the genus: Andean-Patagonian (subgenus *Andina*) and Austral (subgenus *Azorella*). Within the former, the main sections or sub-lineages recognized in all phylogenetic analyses to date were also sampled: Laretia (= Diversifolia sub-lineage), Pectophytum plus Glabratae (= Trifurcata sub-lineage) and Spinosae (= Spinosum sub-lineage). The classification into sections is according to Plunkett and Nicolas (2017) and that of sub-lineages follows Fernández et al. (2017a). Flower buds and open flowers of selected species were stored for embryology, and 10–15 mature fruits per plant (if present) were collected for flow cytometry seed screen analysis (FCSS). In addition, a fresh leaf was

sampled for absolute genome size determination using flow cytometry (FCM). Herbarium vouchers were deposited in BCRU, QCA, LPB and PRC (Supplementary Table 1).

Table 1

Chromosome counts, mean absolute genome size (propidium iodide staining, 2C value in pg) and mean variation coefficient value (mean CV in percentage) of the maternal sporophyte, endosperm ploidy, and embryo:endosperm ratio (Emb:end) of the *Azorella* and *Pozoa* species examined. Only FCSS samples that resulted in two peaks were considered. The infrageneric classification into subgenera and sections follows Plunkett and Nicolas (2017) and Fernández and Calviño (2019). SD – standard deviation, NA – data not available, chromosome counts are taken from published studies (Bell and Constance 1957; Constance et al. 1971, 1976; Moore 1981; Constance 1988).

| Species | 2n | Mean 2C ± SD [pg] | Mean CV ± SD [%] | FCM samples [No.] | Emb:end | Endosperm ploidy level | FCSS samples [No.] | Subgenus | Section |
|---|---|---|---|---|---|---|---|---|---|
| *A. andina* (Phil.) Drude | NA | 8.13 ± 0.52 | 3.03 ± 0.90 | 5 | – | – | NA | Azorella | Huanaca |
| *A. aretioides* DC | NA | 12.14 ± 0.52 | 2.67 ± 0.44 | 9 | 0.393 | 5n | 15 | Andinae | Laretia |
| *A. biloba* (Schltdl.) Wedd. | 16 | 12.02 +. 041 | 2.38 ± 0.79 | 11 | 0.369 | 5n | 18 | Andinae | Laretia |
| *A. boelckei* (Mathias & Constance) G.M. Plunkett & A.N. Nicolas | NA | 8.46 | 2.51 | 1 | – | – | NA | Azorella | Ranunculus |
| *A. burkartii* (Mathias & Constance) G.M. Plunkett & A.N. Nicolas | NA | 8.53 ± 0.04 | 2.94 ± 0.41 | 2 | – | – | NA | Azorella | Ranunculus |
| *A. compacta* Phil. | 16 | 10.48 | 1.85 | 1 | 0.396 | 5n | 1 | Andinae | Laretia |
| *A. corymbosa* (Ruiz & Pav.) Pers. | NA | 3.79 ± 0.12 | 2.48 ± 0.59 | 4 | 0.387 | 5n | 15 | Andinae | Pectophytum |
| *A. crassipes* Phil. | NA | 2.85 ± 0.09 | 1.61 ± 0.50 | 2 | – | – | NA | Andinae | Pectophytum |
| *A. diapensoides* A. Gray | NA | 10.99 ± 0.07 | 1.97 ± 0.17 | 3 | 0.389 | 5n | 4 | Andinae | Laretia |
| *A. lycopodioides* Gaudich. | 16 | 4.4 ± 1.65 | 2.23 ± 0.73 | 9 | – | – | NA | Andinae | Glabratae |
| *A. madreporica* Clos | NA | 6.22 ± 0.12 | 2.03 ± 0.48 | 2 | – | – | NA | Andinae | Laretia |
| *A. microphylla* (Cav.) G.M. Plunkett & A.N. Nicolas | 32 | 10.76 | 3.46 | 1 | – | – | NA | Andinae | Laretia |
| *A. monantha* Clos | 16 | 6.88 ± 0.19 | 2.90 ± 0.93 | 7 | 0.386 | 5n | 9 | Andinae | Laretia |

| Species | 2n | Mean 2C ± SD [pg] | Mean CV ± SD [%] | FCM samples [No.] | Emb:end | Endosperm ploidy level | FCSS samples [No.] | Subgenus | Section |
|---|---|---|---|---|---|---|---|---|---|
| *A. multifida* (Ruiz & Pav.) Pers. | NA | 3.52 ± 0.28 | 1.70 ± 0.04 | 3 | – | – | NA | Andinae | Pectophytum |
| *A. nivalis* Phil. | 16 | 6.83 ± 0.33 | 2.96 ± 1.38 | 6 | 0.408 | 5n | 1 | Andinae | Spinosae |
| *A. pedunculata* (Spreng.) Mathias & Constance | 16 | 3.57 ± 0.17 | 2.39 ± 0.90 | 10 | 0.383 | 5n | 19 | Andinae | Pectophytum |
| *A. prolifera* (Cav.) G.M. Plunkett & A.N. Nicolas | 16,32,48 | 16.07 ± 5.24 | 2.64 ± 0.75 | 11 | 0.399 | 5n | 9 | Andinae | Spinosae |
| *A. trifoliolata* Clos | 16 | NA | NA | 0 | 0.397 | 5n | 12 | Andinae | Laretia |
| *A. trifurcata* (Gaertn.) Pers. | 16 | 3.41 ± 0.22 | 2.58 ± 0.89 | 4 | 0.385 | 5n | 5 | Andinae | Pectophytum |
| *P. coriaceae* Lag. | 20 | 3.06 ± 0.12 | 1.92 ± 0.66 | 3 | 0.684 | 3n | 5 | – | – |
| *P. volcanica* Mathias & Constance | 20 | 3.46 ± 0.89 | 1.60 ± 0.52 | 2 | 0.508 | 4n | 4 | – | – |

# Flow cytometric seed screening

The flow cytometric seed screen (FCSS) followed the protocol of Matzk et al. (2000) modified by Krahulcová and Rotreklová (2010). The FCSS was performed separately for each of 276 mature fruits; analyses of five fruits per individual were conducted whenever possible. The DNA content of seed embryo and endosperm tissues was estimated by flow cytometry using a CyFlow ML instrument (UV LED chip and DAPI as fluorescent dye) to evaluate the ploidy of endosperm. A single mature fruit was chopped together with an appropriate amount of the respective internal standard (according to Temsch et al. 2021); see the section Absolute genome size analysis) in 0.65 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) in a Petri dish. The suspension was filtered through a 42-µm nylon mesh filter and incubated at room temperature for 10 min. The suspension was then mixed with 1 ml of Otto II buffer [0.4 M Na2HPO4·12H2O, β-mercaptoethanol, DAPI (4',6-diaminido-2-fenylindol) fluorescent dye; Otto (1990)]. The relative fluorescence of 3,000 particles was recorded and the resulting histograms (with CV < 5%) were analysed in FloMax software (version 2.4d, Partec, Münster, Germany).

# Embryology

Flower buds and open flowers of four species of *Azorella* (*Azorella aretioides* DC, *A. crassipes* Phil, *A. corymbosa* (Ruiz & Pav.) Pers. and *A. andina* (Phil.) Drude) were fixed and stored in an FAA solution (70% ethanol, 38% formaldehyde, glacial acetic acid in ratio 7:2:1). Fixed flowers were washed in distilled water, dehydrated using 100% ethanol and cleared in methyl salicylate according to Tucker et al. (2003). Ovules were dissected from cleared flowers under an Olympus SZ61 stereo microscope and observed using Nomarski differential interference contrast under an Olympus BX51 light microscope with an Olympus DP72 camera at 1,000× magnification.

# Absolute genome size analysis

To compare the DNA ploidy level and genome size of the embryo against the maternal sporophyte, FCM was conducted using leaf tissue. Absolute genome size was determined using a CyFlow SL instrument (Partec GmbH, Münster, Germany) equipped with a green solid-state laser, with propidium iodide as the fluorescent dye. Sample preparation followed the simplified two-step protocol of Doležel et al. (2007). We used three different internal standards (*Carex acutiformis*, 2C = 0.818 pg, *Bellis perennis*, 2C = 3.38 pg and *Pisum sativum* cv. Ctirad, 2C = 9.09 pg; Temsch et al. 2021) because of the wide range of absolute genomes size in the subfamily Azorelloideae. Only fresh leaf material was used to estimate absolute genome size. Approximately 0.5 $cm^2$ of leaf tissue of each sample and an appropriate amount of the corresponding internal reference standard were chopped together in 0.65 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) in a Petri dish. The suspension was filtered through a 42-µm nylon mesh filter and incubated at room temperature for 10 min. The suspension was then mixed with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$, β-mercaptoethanol, propidium iodide and RNase IIA). The relative fluorescence of 5,000 particles was recorded. Each sample was analysed at least twice on different days, and only analyses not differing by more than 3% were accepted. Histograms were analysed in FloMax software (version 2.4d, Partec, Münster, Germany) and only measurements with a coefficient of variance (CV) below 5% were evaluated.

# Results

Out of a total of 276 seeds examined, two peaks were present in 117 seeds (42.4% of the total) of thirteen species (11 *Azorella* + 2 *Pozoa*), allowing to estimate the ploidy level of the embryo and the endosperm (Table 1, Fig. 2). The mean coefficient of variation (CV) for the embryo and endosperm was 2.2% (range 1.72–3.64) and 1.5% (range 0.74–4.04), respectively. The mean CV for the standard was 2.1% (range 1.24–4.73).

Our flow cytometric seed screen revealed a pentaploid endosperm (5n) in all eleven species of *Azorella* studied (Fig. 3; Table 1). Embryological observations confirmed a tetrasporic embryo sac (*Penaea*-type) and a pentaploid endosperm (5n) in four species of *Azorella* (*Azorella aretioides*, *A. crassipes*, *A. corymbosa* and *A. andina* (Fig. 4). Each tetrasporic embryo sac consisted of 16 nuclei arranged in four polar groups each consisting of three cells (one chalazal cell, two lateral cells and an egg apparatus with two synergids and one egg cell at the micropillar pole) and a central cell with four polar nuclei (Fig. 4). In such embryo sacs, after fertilization the resultant endosperm is pentaploid (5n) in diploid species, as confirmed by the FCSS (Fig. 3).

The two species of *Pozoa* possessed endosperm of different ploidy levels. *Pozoa coriacea* Lag. had a triploid endosperm (3n, embryo:endosperm 2:3, mean 0.684, 4.8%) whereas *P. volcanica* Mathias & Constance formed a tetraploid endosperm (4n, embryo:endosperm ratio 2:4, mean 0.508, 3.8%). The triploid endosperm in *Pozoa coriacea* is consistent not only with a *Drusa*-type embryo sac, but also with a *Polygonum*-type embryo sac.

Absolute genome size was determined in 96 individuals of twenty species of the subfamily Azorelloideae (2C = 1.95–21.02 pg, amounting to 10.77-fold variation; see Table 1). The mean variation coefficient (CV) for the entire dataset was 2.5% (range: 1.23–4.82) and that for the internal reference standards was 2.8% (range: 1.6–4.75) for the internal reference standards. The pattern of absolute genome size variation in *Azorella prolifera* corresponded to DNA polyploidy with three different genome sizes: DNA diploid (2C = 6.84 ± 0.057 pg), DNA tetraploid (2C = 13.12 ± 0.196 pg) and DNA hexaploid (2C = 19.55 ± 0.659 pg), which is consistent with previously published chromosome counts (Constance et al. 1971; Constance 1988) and the formation of endosperm with the corresponding ploidy level. The ploidy and genome size of all maternal sporophytes corresponded to the ploidy level of all embryos without evidence of heteroploidy hybridization or the formation of unreduced gametes.

# Discussion

The present study uses an original methodology that combines broad quantitative flow cytometry seed screening with classical embryological techniques applied to selected species to analyse the type of embryo sac and endosperm ploidy level. This combination allows to efficiently analyse great numbers of samples with FCSS and confirm the type of embryo sac embryologically when appropriate. The latter is particularly important because, for instance, a 2:5 embryo:endosperm ratio without embryological evidence, is generally indicative of pseudogamous apomixis, where endosperm formation requires fertilization of the polar nuclei (Matzk et al. 2000). However, that ratio could also be indicative of sexual reproduction resulting

from double fertilization of the egg cell and a central 4n cell, as present in embryo sacs of the types *Fritillaria*, *Plumbago* or *Penaea* (Kordyum and Mosyakin 2020). FCSS therefore allows fast acquisition and screening of data, and embryology provides confirmation of the type of embryo sac and endosperm variation in representative lineages. Combined, these approaches allow for reliable yet efficient studies, for example, in broad phylogenetic analyses that require extensive sampling.

Within the Apiaceae, embryo sac and endosperm variation has so far been studied in relatively few genera (ca 70, mostly of the subfamily Apioideae; see the sampling discussed in Tseng (1967) or analysed in Henwood and Hart (2001). Based on different systematic studies (Jurica 1922; Håkansson 1923, 1927, 1952; Tseng 1967; Henwood and Hart 2001) all subfamilies except the Azorelloideae have a *Polygonum*-type embryo sac, specifically, monosporic, 8-nucleate and with a 3n endosperm. However, the few Azorelloideae species analysed in those studies differed in the type of embryo sac from the rest of the umbellifers in being tetrasporic and 16-nucleate. This led to the idea that a tetrasporic and 16-nucleate embryo sac is a synapomorphy of the Azorelloideae. Our results, based upon a five-fold increase in the sampling of azorelloid species, support this notion. However, the lack of embryological results for *Pozoa* allows for the possibility that not all azorelloids have a tetrasporic and 16-nucleate embryo sac, given that the 3n endosperm estimated for this species based on FCSS is also compatible with a *Polygonum*-type embryo sac. Endosperm derived from tetrasporic female gametophytes should be favoured over those derived from monosporic female gametophytes because of increased endosperm heterozygosity and endosperm relatedness to the maternal sporophyte in the former (Friedman et al. 2008). This is compatible with a unique transition from monosporic to tetrasporic endosperms, with no reversals, in an ancestor of the Azorelloideae. However, it is also compatible with multiple acquisitions of the tetrasporic condition within the subfamily. A broader sampling of genera within the Azorelloideae is still needed to understand if tetrasporic embryo sacs evolved only once in the ancestor of the Azorelloideae or multiple times within the subfamily.

Within the Azorelloideae, different types of embryo sac and endosperm ploidy levels have been reported (Tseng 1967; Henwood and Hart 2001, this study). *Drusa* and *Bowlesia* have a *Drusa*-type embryo sac (Tseng 1967). In our present study, we examined *Pozoa* for the first time and found a 3n endosperm in *P. coriacea* which is compatible with a *Polygonum*- or *Drusa*-type embryo sac. All *Azorella* species studied so far (Tseng 1967; Henwood and Hart 2001), including those in our study, have a *Penaea*-type female gametophyte. Importantly, the *Penaea*-type differs from both other types of embryo sac in the ploidy level of the resultant endosperm (Maheshwari 1937). A *Polygonum*- or *Drusa*-type embryo sac has two polar nuclei in its central cell, so a 3n endosperm is formed after fertilization between diploids (Friedman 1998; Friedman et al. 2008). By contrast, a *Penaea*-type embryo sac has four polar nuclei which result in a 5n endosperm after fertilization (Maheshwari 1937). Considering our current knowledge on the phylogenetic distribution of the types of embryo sac and endosperm ploidy within the Azorelloideae, and because the *Penaea*-type and 5n endosperm have only been reported for members of *Azorella* that belong to different sub-lineages of the genus, we propose that these characteristics are likely synapomorphies of the whole genus.

According to the ploidy, heterozygosity, and parental conflict hypotheses, higher endosperm ploidy, increased maternal-to-paternal genomic ratios and increased relatedness of the maternal sporophyte to the endosperm contained within its seeds should be favoured over the course of evolution (Friedman et al. 2008). Our results support those predictions; within *Azorella*, a unique transition from a triploid to a pentaploid endosperm was estimated with no reversals. In pentaploid endosperm the contribution of female cells is more than double, and the central cell always includes both alleles of the maternal genotype (Baroux et al. 2002). The female gametophyte is thought to increase the success rate of seed germination and seedling establishment during the colonization of harsher environments in cold alpine habitats (Donoghue and Scheiner 1992; Härdling and Nilsson 2001; Friedman et al. 2008). A higher ploidy of endosperm possibly allows for higher rates of gene transcription (D'Amato 1984). The higher ploidy of endosperm may therefore have the same effect on the success of initial phases of plant development as polyploidization (Soltis et al. 2009). A maternal to paternal genomic ratio of 4:1 is needed for successful endosperm development, which results in a pentaploid endosperm (Carputo et al. 1999). Transitions to pentaploid endosperm (5n) occurred several times in different plant groups such as the Piperaceae, Liliaceae, Plumbaginaceae and Asteraceae (Maheshwari 1937; Haig 1990). In the Apiaceae, the evolution of pentaploid endosperm (5n) may have facilitated the colonization of alpine habitats following the environmental changes that took place at the turn of the Miocene and Pleistocene (Simpson and Todzia 1990; Testolin et al. 2021), and that enabled members of the genus *Azorella* to become some of the most dominant species of alpine vegetation in the Andes. The South American genus *Gunnera* shares with *Azorella* a partially similar history of colonizing South America (Sklenář et al. 2011; Nicolas and Plunkett 2012; Fernández et al. 2017a) and shares the same *Penaea*-type embryo sac

(Virkki 1962; Wilkinson and Wanntorp 2007), supporting the idea of convergence towards higher endosperm ploidy in harsh environments.

As mentioned above, no variation in endosperm ploidy was detected across all lineages of *Azorella* sampled. However, the two species of *Pozoa* exhibited differing embryo:endosperm ratios. *Pozoa coriacea* has a 2:3 embryo:endosperm ratio as determined by FCSS whereas *P. volcanica* exhibits an atypical 2:4 embryo:endosperm ratio. The latter is not compatible with sexual reproduction involving any type of embryo sac described (Maheshwari 1937; Friedman et al. 2008; Kordyum and Mosyakin 2020) as it would imply fertilization of a 3-nucleate central cell to form the endosperm. The only biological explanation left is that the 2:4 embryo:endosperm ratio is the result of autonomous apomixis in *P. volcanica*. An alternative explanation of the 2:4 ratio, a G2 peak, is unlikely because we have not found any G2 peak for embryo tissue during our FCSS of the entire Azorelloideae. Autonomous apomixis results from apomeiosis leading to an unreduced embryo sac and development of the embryo and endosperm without fertilization (Kaushal et al. 2019). Apomixis combines the benefits of seed dispersal, increasing the potential for long-distance dispersal, and reproductive assurance in the introduced area without the need for pollinators (Hörandl 2006; Hojsgaard and Hörandl 2015). Population-level studies within *Pozoa* hypothesize that *P. volcanica* arose through a process of progenitor–derivative speciation from *P. coriacea* (López et al. 2011). This scenario is supported not only by phylogeny and populations genetics, but also by biogeographical and ecological observations that indicate that *P. volcanica* was able to colonize a new and unique ecological zone with volcanic soils in the regions of Araucanía (Chile) and Neuquén (Argentina; López et al. 2011; Martínez and Calviño 2019) The fact that both species are outcrossing diploids (López et al. 2011) does not contradict our hypothesis of autonomous apomictic reproduction in *P. volcanica*, given that apomictic plants are not obligate apomicts and most of them produce low levels of sexually derived individuals besides clonally derived ones (Hojsgaard and Hörandl 2015). Our results therefore support the hypothesis of López et al. (2011) and adds that apomixis was probably the driver of the range expansion and divergence of *P. volcanica*. More analyses of these species are needed to confirm these hypotheses.

# Conclusions

Variation in the type of embryo sac and endosperm ploidy have probably shaped the evolution of the different lineages of Azorelloideae in the southern Andes. The *Penaea*-type embryo sac and the formation of a pentaploid endosperm (5n) are putative synapomorphies of *Azorella.*

Higher ploidy of endosperm does not necessarily indicate apomixis, and a different type of embryo sac should always be kept in mind as an alternative explanation when interpreting FCSS results. It has been shown that FCSS can serve as a guide for the identification of other types of embryo sac, especially tetrasporic ones, in which the resulting endosperm has a higher ploidy level than triploid. Flow cytometry provides new insights into endosperm ploidy level, enabling truly extensive sampling.

# Declarations

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Data availability

Primary FCM data and figures of embryological observation are supplemented.

### Funding

This work was supported by the Grant Agency of the Czech Republic (project No. 17-12420S), the Grant Agency of Charles University (project No. 1554218) and the following institutions in Argentina: Agencia Nacional de Promoción Científica y Tecnológica PICT 2019-007700, CONICET PIP 112-201301-00357 and Universidad Nacional del Comahue PIN B205.

### Authors' contributions

Carolina I. Calviño, Petr Sklenář and Tomáš Urfus conceived the ideas; Jan Ptáček, Petr Sklenář, Tomáš Urfus and Carolina I. Calviño collected the samples; Jan Ptáček, Tomáš Urfus, Romana Urfusová and Jan Pinc performed the laboratory measurements; Carolina I. Calviño determined the species; and Jan Ptáček analysed the data and led the writing of this article with assistance from Carolina I. Calviño, Petr Sklenář and Tomáš Urfus.

**Acknowledgements**

We are grateful to Lenka Macková, Veronika Konečná, Katya Romoleroux, Romina Vidal-Russell for their assistance. Fred Rooks kindly improved the English. The necessary research permit was issued by the Ministerio del Ambiente, Ecuador (No. MAE-DNB-CM-2018-0082), the Ministerio de medio ambiente y agua, Bolivia (No. 026/09) and the Administración de Parque Nacionales, Argentina (No. 1621).

# References

1. Andersson L, Kocsis M, Eriksson R (2006) Relationships of the genus *Azorella* (Apiaceae) and other hydrocotyloids inferred from sequence variation in three plastid markers. Taxon 55:270–280. https://doi.org/10.2307/25065577
2. Baroux C, Spillane C, Grossniklaus U (2002) Evolutionary origins of the endosperm in flowering plants. Genome Biol 3:1–5
3. Bell CR, Constance L (1957) Chromosome numbers in Umbelliferae. Am J Bot 44:565–572. https://doi.org/10.2307/2438928
4. Benton MJ, Wilf P, Sauquet H (2022) The Angiosperm Terrestrial Revolution and the origins of modern biodiversity. New Phytol 233:2017–2035. https://doi.org/https://doi.org/10.1111/nph.17822
5. Calviño CI, Fernández M, Martínez SG (2016) Las Especies de *Azorella* (Azorelloideae, Apiaceae) con distribució extra-Argentina. Darwiniana 4:57–82. https://doi.org/10.14522/darwiniana.2016.41.681
6. Caparelli KF, Peruzzi L, Cesca G (2006) A comparative analysis of embryo-sac development in three closely-related *Gagea* species (Liliaceae), with some considerations on their reproductive strategies. Plant Biosyst 140:115–122
7. Carputo D, Monti L, Werner JE, Frusciante L (1999) Uses and usefulness of endosperm balance number. Theor Appl Genet 98:478–484. https://doi.org/10.1007/s001220051095
8. Constance L (1988) Umbelliferae. In: Correa MN (ed) Flora Patagónica. Colección Científica del Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, pp 310–379
9. Constance L, Chuang T, Bell CR (1971) Chromosome Numbers in Umbelliferae. IV. Am J Bot 58:577–587. https://doi.org/10.1002/j.1537-2197.1971.tb10007.x
10. Constance L, Chuang T, Bell CR (1976) Chromosome numbers in Umbelliferae. V. Am J Bot 63:608–625. https://doi.org/10.1002/j.1537-2197.1976.tb11849.x
11. D'Amato F (1984) Role of polyploidy in reproductive organs and tissues. In: Embryology of angiosperms. Springer, pp 519–566
12. Doležel J, Greilhuber J, Suda J (2007) Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
13. Donoghue MJ, Scheiner SM (1992) The evolution of endosperm: a phylogenetic account. In: Wyatt R (ed) Ecology and evolution of plant reproduction. Chapman & Hall, New York, pp 356–389
14. Ebert I, Greilhuber J (2005) Developmental switch during embryo sac formation from a bisporic mode to the tetrasporic *Fritillaria* type in *Hyacinthoides vincentina* (Hoffmannsegg & Link) Rothm.(Hyacinthaceae). Acta Biol Cracov Bot 47:179–184
15. Fagerlind F (1938) Wo kommen tetrasporische, durch drei Teilungsschritte vollentwickelte Embryosäcke unter den Angiospermen vor?
16. Fernández M, Calviño CI (2019) Nueva clasificación infragenérica de *Azorella* (Apiaceae, Azorelloideae) y sinopsis del subgénero *Andinae*. Darwiniana, nueva Ser 7:289–304

17. Fernández M, Ezcurra C, Calviño CI (2016) Morphology, fruit anatomy and taxonomy of the South Andean genus *Laretia* (Azorelloideae, Apiaceae). Syst Bot 41:807–812. https://doi.org/10.1600/036364416X692370
18. Fernández M, Ezcurra C, Calviño CI (2017a) Molecular Phylogenetics and Evolution Chloroplast and ITS phylogenies to understand the evolutionary history of southern South American *Azorella*, *Laretia* and *Mulinum* (Azorelloideae, Apiaceae). Mol Phylogenet Evol 108:1–21. https://doi.org/10.1016/j.ympev.2017.01.016
19. Fernández M, Ezcurra C, Calviño CI (2017b) Revisión taxonómica del género sudamericano *Mulinum* (Azorelloideae, Apiaceae). An del Jardín Botánico Madrid 74:1–31
20. Fernández M, Ezcurra C, Calviño CI (2017c) Species limits and morphometric and environmental variation within the South Andean and Patagonian *Mulinum spinosum* species-group (Apiaceae-Azorelloideae). Syst Biodivers 15:489–505. https://doi.org/10.1080/14772000.2016.1273975
21. Fernandez M, Martínez SG, Calviño CI (2020) Typifications of 31 names in *Azorella* (Azorelloideae, Apiaceae) from South America. Phytotaxa 433:135–144. https://doi.org/10.11646/phytotaxa.433.2.4
22. Friedman WE (1998) The evolution of double fertilization and endosperm: an" historical" perspective. Sex Plant Reprod 11:6–16
23. Friedman WE, Madrid EN, Williams JH (2008) Origin of the fittest and survival of the fittest: relating female gametophyte development to endosperm genetics. Int J Plant Sci 169:79–92. https://doi.org/10.1086/523354
24. Haig D (1990) New perspectives on the angiosperm female gametophyte. Bot Rev 56:236–274. https://doi.org/10.1007/BF02858326
25. Håkansson A (1923) Studienüber die Entwicklungsgeschichite der Umbelliferae. Acta Univ Lund 18:1–120
26. Håkansson A (1952) Seed development in *Bowlesia tenera*. Bot Not 1:33–45
27. Håkansson A (1927) Der sechzehnkernige Embryosack von *Azorella trifurcata* Gaertn. Hook. Ber Deutsch Bot Ges 45:654–664
28. Härdling R, Nilsson P (2001) A model of triploid endosperm evolution driven by parent-offspring conflict. Oikos 92:417–423. https://doi.org/10.1034/j.1600-0706.2001.920303.x
29. Henwood MJ, Hart JM (2001) Towards an understanding of the phylogenetic relationships of Australian Hydrocotyloideae (Apiaceae). Edinburgh J Bot 58:269–289
30. Hojsgaard D, Hörandl E (2015) Apomixis as a facilitator of range expansion and diversification in plants. In: Evolutionary biology: biodiversification from genotype to phenotype. Springer, pp 305–327
31. Hörandl E (2006) The complex causality of geographical parthenogenesis. New Phytol 171:525–538. https://doi.org/10.1111/j.1469-8137.2006.01769.x
32. Jurica HS (1922) A morphological study of the Umbelliferae. Bot Gaz 74:292–307
33. Kaushal P, Dwivedi KK, Radhakrishna A, et al (2019) Partitioning apomixis components to understand and utilize gametophytic apomixis. Front Plant Sci 10:256
34. Kordyum EL, Mosyakin SL (2020) Endosperm of Angiosperms and Genomic Imprinting. Life 10:1–20
35. Krahulcová A, Rotreklová O (2010) Use of flow cytometry in research on apomictic plants. Preslia 82:23–39
36. López P, Tremetsberger K, Kohl G, Stuessy T (2011) Progenitor–derivative speciation in *Pozoa* (Apiaceae, Azorelloideae) of the southern Andes. Ann Bot 109:351–363. https://doi.org/10.1093/aob/mcr291
37. Maheshwari P (1937) A critical review of the types of embryo sacs in angiosperms. New Phytol 36:359–417
38. Martinez S (1989) El género *Azorella* (Apiaceae-Hydrocotyloideae) en la Argentina. Darwiniana 1:139–178
39. Martínez SG, Calviño CI (2019) Apiaceae. In: Zuloaga FO, Belgrano MJ (eds) Flora Vascular de la República Argentina 20 (2). Talleres Trama S.A., Buenos Aires, pp 1–122
40. Matzk F, Meister A, Schubert I (2000) An efficient screen for reproductive pathways using mature seeds of monocots and dicots. Plant J 21:97–108. https://doi.org/10.1046/j.1365-313x.2000.00647.x
41. Moore DM (1981) Chromosome numbers of Fuegian angiosperms. Bol Soc Brot 53:995–1012

42. Nicolas AN, Plunkett GM (2009) The demise of subfamily Hydrocotyloideae (Apiaceae) and the re-alignment of its genera across the entire order Apiales. Mol Phylogenet Evol 53:134–151. https://doi.org/10.1016/j.ympev.2009.06.010
43. Nicolas AN, Plunkett GM (2012) Untangling generic limits in *Azorella*, *Laretia*, and *Mulinum* (Apiaceae: Azorelloideae): Insights from phylogenetics and biogeography. Taxon 61:826–840. https://doi.org/10.1002/tax.614008
44. Nuñez CI, Aizen MA, Ezcurra C (1999) Species associations and nurse plant effects in patches of high-Andean vegetation. J Veg Sci 10:357–364. https://doi.org/10.2307/3237064
45. Otto F (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Methods in cell biology. Elsevier, pp 105–110
46. Plunkett GM, Nicolas AN (2017) Assessing *Azorella* (Apiaceae) and its allies: Phylogenetics and a new classification. Brittonia 69:31–61. https://doi.org/10.1007/s12228-016-9446-0
47. Rangan P (2020) Endosperm variability: from endoreduplication within a seed to higher ploidy across species, and its competence. Seed Sci Res 30:173–185
48. Simpson BB, Todzia CA (1990) Patterns and processes in the development of the high Andean flora. Am J Bot 77:1419–1432. https://doi.org/10.1002/j.1537-2197.1990.tb12552.x
49. Sklenář P (2009) Presence of cushion plants increases community diversity in the high equatorial Andes. Flora-Morphology, Distrib Funct Ecol Plants 204:270–277
50. Sklenář P, Dušková E, Balslev H (2011) Tropical and temperate: evolutionary history of páramo flora. Bot Rev 77:71–108. https://doi.org/10.1007/s12229-010-9061-9 Tropical
51. Soltis DE, Albert VA, Leebens-Mack J, et al (2009) Polyploidy and angiosperm diversification. Am J Bot 96:336–348
52. Stephens EL (1909) The embryo-sac and embryo of certain Penaeaceae. Ann Bot 23:363–378
53. Subba Rao AM (1937) A note on the development of the female gametophyte of some Malpighiaceae and polyembryony in *Hiptage madablota*. Curr Sci 6:280–282
54. Temsch EM, Koutecký P, Urfus T, et al (2021) Reference standards for flow cytometric estimation of absolute nuclear DNA content in plants. Cytom Part A 1–15. https://doi.org/0.1002/cyto.a.24495
55. Testolin R, Attorre F, Borchardt P, et al (2021) Global patterns and drivers of alpine plant species richness. Glob Ecol Biogeogr 30:1218–1231
56. Tseng CC (1967) Anatomical studies of flower and fruit in the Hydrocotyloideae (Umbelliferae)
57. Tucker MR, Araujo A-CG, Paech NA, et al (2003) Sexual and apomictic reproduction in *Hieracium* subgenus *Pilosella* are closely interrelated developmental pathways. Plant Cell 15:1524–1537. https://doi.org/10.1105/tpc.011742
58. Virkki N (1962) Meiosis and Development of the Embryo Sac in *Gunnera insignis* (Oersted) DC (Halorhagaceae). J Agric Univ Puerto Rico 46:254–268
59. Wilkinson HP, Wanntorp L (2007) Gunneraceae. In: Flowering Plants· Eudicots. Springer, pp 177–183
60. Williams JH, Friedman WE (2002) Identification of diploid endosperm in an early angiosperm lineage. Nature 415:522–526. https://doi.org/10.1038/415522a

# Figures

### Figure 1

Localities of *Azorella* sampled across the latitudinal gradient in South America (30 localities in total). A – tropical Andes (Ecuador, 9 localities), B – (sub)tropical Andes (Bolivia, 7 localities), C – temperate Andes (Argentina, 15 localities). For details on the locations of populations sampled, see Supplementary Tab. 1. The background map is based on freely available vector and raster data from naturalearthdata.com.

**Figure 2**

Flow-cytometric histograms of the relative DNA content of DAPI-stained nuclei isolated from seeds of A – *Azorella corymbosa* (PS 15841, 2:5), B – *A. prolifera* (JP 345, 2:5), C – *Pozoa coriacea* (JP 382, 2:3), D – *P. volcanica* (JP 53, 2:4). X – internal standard *Carex acutiformis*, P – internal standard *Pisum sativum* cv. Ctirad, Em – embryo, En – endosperm, G2 – G2 phase of the standard.

**Figure 3**

Embryo:endosperm ratios of *Pozoa* and the main sections of *Azorella* subgenus Andinae in relation to the absolute genome size (2C, pg). All three sections have an embryo:endosperm ratio of 2:5. The ratio value for *Pozoa coriacea* is 2:3 and that for *P. volcanica* is 2:4.

**Figure 4**

Fully mature tetrasporic embryo sacs of the *Penaea*-type with four polar nuclei. A – *A. crassipes* (JP 107), B – *A. andina* (JP 12), C – *A. corymbosa* (PS 15841), D – *A. aretioides* (PS 15883). A – antipodal, E – egg cell, EA – egg apparatus (containing two synergids and one egg or egg-like cell), PN – polar nuclei, S – synergid. Two egg apparatuses are usually in different planes of focus, so they are not visible in the photographs. The chalazal pole is up, and the micropylar pole is down. Scale bar = 20 μm.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PtaceketalSupplementaryTable1final.xlsx