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COMPLEX GEOGRAPHICAL DISTRIBUTION OF PLOIDY LEVELS IN *POLYLEPIS AUSTRALIS* (ROSACEAE), AN ENDEMIC TREE LINE SPECIES IN ARGENTINA

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Premise of research. The geographical distribution of ploidy levels provides insights into evolutionary pathways. For the subtropical tree line species *Polylepis australis* (Rosaceae), we tested the hypotheses that (1*a*) incidence of polyploidy is higher in the northern parts than in the southern parts of the species range due to the presence of related species that might favor hybridization (allopolyploidy), (1*b*) incidence of polyploidy is higher in the southern part of the range because the species here presumably reaches the limit of its environmental tolerance (autopolyploidy), and (2) ploidy levels increase with elevation, as polyploids are believed to perform better in stressful environments.

Methodology. We used flow cytometry to assess the ploidy levels of 361 individuals from 27 populations across most of the distribution range of the species in two disjunct Argentinean high mountain regions.

Pivotal results. The northern stands had lower ploidy levels (diploid) than the southern populations, in which we found mainly tetraploids intermixed with diploids, triploids, and a single hexaploid. We did not find any environmental correlates to the geographical distribution of ploidy levels.

Conclusions. Polyploidy appears to have arisen in *P. australis* via autopolyploidy, either twice in different parts of the range or, more likely, once followed by long-distance dispersal. This is also supported by amplified fragment length polymorphism (AFLP) data from a previous study that confirmed higher numbers of AFLP fragments and private bands in the southern populations. The checkerboard distribution of ploidy levels in the southern Sierras de Córdoba may represent a time capture of the spread of the polyploid condition. We propose that polyploidy represents a key factor to understanding the high morphological variation in *P. australis* and should be taken into account in ongoing reforestation activities.

Keywords: Andes, autopolyploidy, *Polylepis*, Rosaceae.

Introduction

Polyploidy, the multiplication of entire genomes, has played a pivotal role in the diversification and evolution of plants (Stebbins 1950; Rieseberg and Willis 2007; Soltis et al. 2009). Polyploidization may lead to immediate reproductive isolation between forms of different ploidy levels, opening opportunities for genetic divergence and speciation (Vamosi and Dickinson 2006; Soltis et al. 2007, 2009; Wood et al. 2009). In other cases, ploidy levels mix to produce swarms of individuals with variable ploidy levels and genome sizes (e.g., Talent and Dickinson 2007). Polyploidy has historically been considered to be associated with higher tolerances to environmental stress (Stebbins 1947; Grant 1981), and polyploid taxa are relatively more abundant than diploids in arctic and alpine environments (Stebbins 1985; Brochmann et al. 2004; Hijmans et al. 2007). As such, polyploidization is often considered to play a crucial role in the diversification of plants, especially in mountain habitats (Bierzychudek 1985; Burnier et al. 2009). However, more recent studies have failed to support this generalization, and some authors consider that the geographical and ecological distribution of polyploidy is unpredictable (Hadač 1989; Otto and Whitton 2000; Husband et al. 2013).

The genus *Polylepis* Ruiz & Pav. (Rosaceae) consists of ~30 species growing in Andean montane forests and tree line habitats from Venezuela to central Argentina (Kessler and Schmidt-Lebuhn 2006; Schmidt-Lebuhn et al. 2007). *Polylepis* is one of relatively few wind-pollinated genera in the Rosaceae (Schmidt-Lebuhn et al. 2007; Seltmann et al. 2009), and species of *Polylepis* are known to hybridize with each other (Simpson 1986; Romoleroux 1996; Schmidt-Lebuhn et al. 2006; Segovia et al. 2012). The genus has further been shown to include both diploid and polyploid (tetra- and octoploid) taxa

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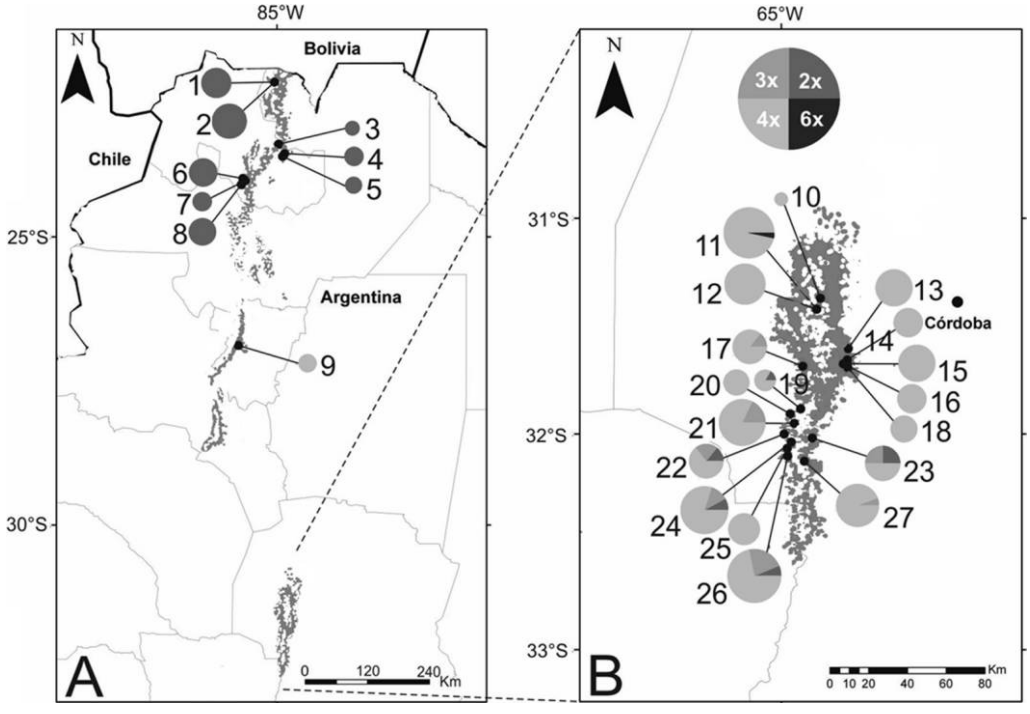

Fig. 1 Location of our study sites (black circles) and pie charts reflecting the proportion of ploidy levels found at each *Polylepis australis* site in Argentina (*A*) and the Sierras de Córdoba (*B*). Pie chart size is proportional to the number of individuals sampled. Numbers next to the pie charts refer to those in table 1. The overall distribution of *P. australis* is indicated by the shaded area (redrawn from Renison et al. 2013).

(Schmidt-Lebuhn et al. 2010). Although the study of Schmidt-Lebuhn et al. (2010) included maximally four samples per species, two species, *P. australis* Bitt. and *P. pauta* Hieron., were inferred to include both diploid and polyploid individuals, suggesting that ploidy variation may occur within species in this genus. More recently, extensive within-species variation of ploidy level as well as aneuploid series of chromosome numbers have been found in the Ecuadorian populations of *P. pauta*, *P. racemosa* Ruiz & Pav., and *P. sericea* Wedd. (Segovia et al. 2012).

In this study, we analyzed ploidy levels in *P. australis*, the southernmost species of the genus. This tree line species is endemic to Argentina, where it occurs in two disjunct regions, the eastern Andean slope of northwestern Argentina and the isolated Sierras de Córdoba in the center of the country (Hensen et al. 2011). These two range parts are separated by a low-lying and arid region that is currently unsuitable for *Polylepis* but that may have supported *Polylepis* stands during cold and humid glacial periods (Ab'Sáber 1977; Luti et al. 1979; Iriondo and García 1993). The total distribution of the species extends over ~1200 km from north to south. About 90% of the distribution of *P. australis* lies between 1500 and 3500 m elevation, with single individuals found as low as 900 m in the Sierras de Córdoba (Marcora et al. 2008). In the north of its range, *P. australis* co-occurs or grows in close proximity to four other species of *Polylepis*, whereas in the Sierras de Córdoba, it is the only species of the genus (Hensen et al. 2011). Studies on the mating system of *P. australis* have revealed high pollen viability and longevity and high effective pollen flow (Seltmann et al. 2007, 2009). Fruits are single-seeded nutlets that are dispersed over short distances by wind.

In order to explore in more detail the geographical distribution of ploidy levels in *P. australis*, we analyzed ploidy levels of 361 individuals from a total of 27 populations from its entire distribution area using flow cytometry. We specifically asked whether (*a*) levels of ploidy vary between the northern and southern distribution areas due to differences in *Polylepis* species diversity and (*b*) climatic and/or topographical characteristics explain the geographical patterns found. Our hypotheses were that (1*a*) incidence of polyploidy should be higher in the northern parts than in the southern parts of the range of the species due to the presence of four other *Polylepis* species in the northern part that might favor hybridization (allopolyploidy), (1*b*) incidence of polyploidy may be higher in the southern part of the range of the species because the genus *Polylepis* here reaches its southernmost limit and presumably the limit of its environmental tolerance (autopolyploidy), and (2) ploidy levels increase with increasing elevation as polyploids are suspected to have better performance in stressful environments. This is the first detailed study on spatial occurrence of polyploidy in a tree line species, covering much of the distribution range.

Material and Methods

Field Sampling

We sampled 27 populations of *Polylepis australis* in two fieldwork phases (fig. 1; table 1). Between July and August

Table 1

Elevation, Coordinates, and Numbers of Individuals of Different Ploidy Levels Recorded in the 27 Study Populations That Are Ordered from North to South

				No. individuals sampled in ploidy class				
Population	Elevation (m)	Lat. (°S)	Long. (°W)	Total	2×	3×	4×	6×
1	3545	22.43	65.07	12	12	...	...	...
2	3460	22.43	65.07	17	17	...	...	...
3	2460	23.40	64.90	2	2	...	...	...
4	2520	23.62	64.92	5	5	...	...	...
5	2600	23.63	64.90	4	4	...	...	...
6	2280	24.10	65.50	11	11	...	...	...
7	2190	24.12	65.51	5	5	...	...	...
8	2180	24.12	65.51	9	9	...	...	...
9	2340	26.96	65.78	3	...	...	3	...
10	1980	31.39	64.79	2	...	...	2	...
11	1940	31.40	64.80	30	...	...	29	1
12	2160	31.41	64.81	21	...	...	21	...
13	1770	31.60	64.71	18	...	...	18	...
14	1900	31.61	64.73	11	...	...	11	...
15	2020	31.62	64.74	18	...	...	18	...
16	2050	31.62	64.75	12	...	...	12	...
17	1850	31.65	64.90	14	...	2	12	...
18	1300	31.67	64.68	9	...	...	9	...
19	2160	31.84	64.88	6	1	...	5	...
20	2090	31.86	64.90	10	...	...	10	...
21	2330	31.88	64.91	28	...	5	23	...
22	2340	31.90	64.92	14	2	3	9	...
23	1680	31.94	64.83	12	3	3	6	...
24	1930	31.97	64.94	25	2	3	20	...
25	1480	31.98	64.96	13	...	...	13	...
26	1960	32.01	64.95	32	2	7	23	...
27	1800	32.06	64.88	18	...	1	17	...
Sums	...	...	...	361	75	24	261	1

2010, we sampled nine sites in northwestern Argentina and seven sites in the Sierras de Córdoba. Because the latter area turned out to show a complex geographical distribution of ploidy levels, in a second field phase (February 2011), we sampled an additional 10 sites in the Sierras de Córdoba, focusing on parts of the mountain range not included in the previous survey. Because we needed live plants for flow cytometry (previous analyses with silica-dried material having failed; A. Kühn et al., unpublished data; J. Suda, personal communication), in most stands we dug out 15–35 young plants and cultivated them in plastic bags at the Ing. Arturo Pagliani field station in Quebrada del Condorito National Park, Córdoba. Leaves were then collected a few days prior to laboratory analyses and stored between humid paper towels in plastic boxes at ~8°C. At sites visited up to 10 d prior to laboratory work, we directly collected leaves in the field and stored them as detailed above. Because some of the cultivated plants died and because technical problems during laboratory work led to unexpected delays during which plant material deteriorated, we ultimately included 2–32 individuals per population in the analyses, resulting in a total of 361 individuals sampled.

Flow Cytometry

Leaves of the first field phase were analyzed at Plant Cytometry Services in Schijndel, the Netherlands, and those of the second field phase at the Instituto de Botánica del Nordeste in Corrientes, Argentina. Analyses followed the protocol described in Schmidt-Lebuhn et al. (2010) with slight modifications. Leaves were first finely chopped with a sharp razor blade in an ice-cold buffer in a plastic petri disc. As buffer, we used Cystain UV precise P (no. 05-5002) from Partec (Münster, Germany) enriched with 0.1% dithiothreitol and 1.0% PVP-40 to reduce the noise in the DNA histograms. For dying, we used 4′,6′-diamidino-2-phenylindole, which has specific DNA-binding properties with preference for adenine-thymine-rich sequences. After chopping, the buffer (~2 mL), containing cell constituents and large tissue remnants, was passed through a nylon filter of 50-μm mesh size. The samples were then run through a CyFlow ML (Partec) using the dichroic mirrors TK 420 and TK 560 and the emission filter GG 435. On average, 3124 ± 234 (mean ± SD) nuclei were measured per sample. Data were analyzed with FloMax, version 2.4 d (Partec). As internal size standard, we used *Ilex crenata* (2.16 pg/2C) in all samples. In the case of triploid individuals, the size standard coincided with the C values of the *Polylepis* plants (2.09–2.24 pg/2C), hampering the specific measurement of the C values of the latter. However, since our aim was not to obtain absolutely precise C values but rather only assignment to ploidy classes, this did not create major problems for our study. Analyses in Argentina followed the same protocol using a Partec PA II flow cytometer, with the detector operating at 355 nm. Data analysis was performed using PA II's Partec FloMax soft-

ware, which includes a peak-finding option. Because parts of the live plants were already quite degraded at the time of analyses and because we used two different laboratories with different analytical machines (but similar protocols) for analyses, we expected some uncertainty in the actual C values. However, values fell into such discrete classes (as previously found by Schmidt-Lebuhn et al. 2010) that assignment to ploidy classes was unambiguous.

Data Analysis

We first tested whether ploidy levels differed between populations in the Andes versus the Sierras de Córdoba using a *G*-test with a 2 × 2 contingency table. Because there was basically no variation in ploidy levels in the Andean part of the range, the following analyses were restricted to populations from the Sierras de Córdoba. Here, to relate the distribution of ploidy levels in different populations of *P. australis* to geographical and ecological parameters, we used elevations as measured in the field with GPS and BioClim climate layers from WorldClim (Hijmans et al. 2005), selecting the highest available resolution grid (30″). Because many of the climate layers in WorldClim covary, we selected the following 10 parameters: BIO1, mean annual temperature; BIO5, maximum temperature of the warmest month; BIO6, lowest temperature of the coldest month; BIO10, mean temperature of the warmest quartal; BIO11, mean temperature of the coldest quartal; BIO12, mean annual precipitation; BIO13, precipitation of the wettest month; BIO14, precipitation of the driest month; BIO16, precipitation of the wettest quartal; and BIO17, precipitation of the driest quartal. We then calculated the percentages of diploid and polyploid individuals per population and used simple linear regression models to explore the relationship of the environmental factors on these percentages. Although there was still covariance among some BioClim factors, we did not consider that this influenced our analysis because we did not find any significant relationships between them and ploidy levels (see "Results"), and covariance would have been problematic only if we had found significant relationships.

Results

Using *Ilex crenata* as a standard, we quantified the genome size of an individual of *Polylepis australis* at 2.88 pg/2C, which closely corresponds to the value of 2.98 ± 0.06 pg/2C (mean ± SE) reported for tetraploid *P. australis* by Schmidt-Lebuhn et al. (2010). Using these values as reference, among the 361 individuals analyzed, we classified 75 individuals (20.9%) as diploid (1.44–1.54 pg/2C), 24 (6.7%) as triploid (2.09–2.24 pg/2C), 261 (72.1%) as tetraploid (2.84–2.97 pg/2C), and a single one (0.3%) as hexaploid (4.15 pg/2C; table 1). There were no noticeable differences in values obtained at the two different laboratories in which the analyses were conducted, so we do not hesitate to combine the data from the two field phases.

Geographically, in the eight northernmost populations, we found only diploid plants, whereas the southernmost Andean population was composed of tetraploids only. All populations from the Sierras de Córdoba were dominated by tetraploid plants (table 1; fig. 1). In nine of them, we recorded only tetraploid plants, whereas one population included both di- and tetraploid plants; three populations tri- and tetraploids; four populations di-, tri, and tetraploids; and one population tetraploids and a single hexaploid plant. Within the Sierras de Córdoba, diploid plants were found in only the western and southern parts of the Sierras, whereas in the northeast, we found only polyploid plants.

A *G*-test based on a 2 × 2 contingency table confirmed the obvious difference in frequency of diploids versus polyploids in the Andes compared to the Sierras de Córdoba ($G = 256.3$, $P < 0.001$). Restricting the analyses to the populations from the Sierras de Córdoba, we found no significant relationships between the percentage of diploid or polyploid individuals per population and elevation (linear regression, $r = 0.12$, $P = 0.66$) or any of the 10 BioClim variables selected from WorldClim ($r = 0.04$–0.33, $P = 0.18$–0.87).

Discussion

In this detailed study of the geographical distribution of ploidy levels in a species of *Polylepis*, we found a high diversity and geographical complexity of ploidy levels. Because our C values fell in distinct classes separated by large gaps, as previously found by Schmidt-Lebuhn et al. (2010), we are confident that the assignation to ploidy levels was unambiguous. However, because a portion of the studied plants was already fairly old and because we used two different laboratories for analyses, we did not attempt to assess any minor variation within ploidy classes. We might thus have been unable to detect small variation in C values as reported for three other species of *Polylepis* in Ecuador that had different aneuploid chromosome numbers within ploidy levels and, hence, variability of nucleus sizes (Segovia et al. 2012).

Schmidt-Lebuhn et al. (2010) were the first to document different ploidy levels in *Polylepis*, finding mainly what they interpreted as diploid (2n = ~40) and tetraploid (2n = ~80) taxa along with one octoploid species (*Polylepis racemosa*). More recently, Segovia et al. (2012) have studied the karyology of four species of *Polylepis* in Ecuador in more detail, interpreting individuals with 2n = 42 as hexaploids. While more detailed analyses will be necessary to settle the ultimate ploidy levels of *P. australis*, we here call plants with the lowest ploidy level diploids and those with higher genome sizes triploids, tetraploids, and hexaploids. We consider this interpretation to be valid because the genome sizes we documented almost perfectly fit the relative sizes for these ploidy levels (1 : 1.45; 1 : 1.94; 1 : 2.88) without any intermediate measurements that one might expect in mixtures of even higher ploidy levels.

High diversity of ploidy levels is not unusual in members of Rosaceae, among which at least 32 of 85 genera show polyploidy (Dickinson et al. 2007), and this has been studied in detail in, for example, *Crataegus* (Talent and Dickinson 2007; Lo et al. 2010) and *Sorbus* (Robertson et al. 2010; Pellecier et al. 2012). On the other hand, hexaploids are not common in the family, having so far been recorded only in *Cotoneaster*, *Crataegus*, *Malus* (Dickinson et al. 2007), and *Polylepis* (Segovia et al. 2012). However, not all species of *Polylepis* appear to have variable ploidy levels. Schmidt-Lebuhn et al. (2010) found constant ploidy levels for 9 of 11 species based on up to four individuals per species, Segovia et al. (2012) found the

same for *P. incana* in a more exhaustive sampling, and our sampling of several populations of Argentinian *P. hieronymi* Pilg. (diploid, $N = 52$ individuals from three sites) and *P. tomentella* Wedd. ssp. *tomentella* (tetraploid, $N = 43$, three sites; A. Kühn et al., unpublished data) also documents constant ploidy in these two species.

We found a clear geographic pattern in the distribution of ploidy levels in *P. australis*. Most of the Andean populations are diploid, except the southernmost one, in which we found only tetraploid individuals, albeit with a low sample size ($N = 3$). More detailed sampling of these southern populations and of geographically intermediate populations between these and the northern diploid ones would be necessary to document the extent of tetraploid populations in the Andean range of the species and the zone of contact or overlap between diploid and tetraploid populations. In the geographically isolated Sierras de Córdoba, we found mainly tetraploid plants in the northeastern part of the Sierras and mixed di-, tri-, and tetraploid populations in the southwest. There are no evident environmental differences (elevation, climate, geology) between these regions of the Sierras. Polyploids are known to generally maintain higher levels of heterozygosity than their diploid progenitors (Soltis and Soltis 2000). In accordance, Hensen et al. (2011), using amplified fragment length polymorphisms (AFLPs) on a sample of *P. australis* populations that partially overlapped with ours, reported lower within-population genetic diversity in the northernmost Andean populations compared to those of the Sierras de Córdoba. In addition, the average number of polymorphic bands in the diploid northern Andean range was lower than those of the two groups dominated by tetraploids, the southern part of the Andean range and the southern populations of the Sierras de Cordoba (185, 211, and 207, respectively). This is in accordance to the view that relative to diploids, polyploids generally produce higher numbers of AFLP fragments (Meudt and Clarke 2007).

These patterns raise a number of questions, many of which call for further research. The first question centers on the mode of polyploidization. We consider it likely that polyploidy in *P. australis* resulted from autopolyploidization, for two reasons. First, everywhere where we recorded polyploid individuals, *P. australis* is the only species of *Polylepis* present. Second, the extremely low frequencies of private AFLP bands found in the diploid northern Andean range (0.5%), the southern part of the Andean range (0%), and the southern populations of the Sierras de Cordoba (0.5%) in the study of Hensen et al. (2011) indicate that there was no additional parental gene pool involved in the evolution of the tetraploids. Similarly low frequencies of private AFLP fragments have been reported for the autotetraploid *Ranunculus kuepferi* by Cosendai et al. (2011). Thus, while other scenarios such as allopolyploid formation followed by long-distance dispersal or extinction of some parent populations are also conceivable, on current evidence we consider an autopolyploid origin to be more likely. This hypothesis requires testing by molecular phylogenies including both diploid and tetraploid individuals.

The second issue concerns the geographical checkerboard distribution of ploidy levels in *P. australis*. Polyploidy has historically often been considered to be associated with higher tolerances to environmental stress (Stebbins 1947; Grant 1981) and polyploid taxa relatively more abundant than diploids in arctic and alpine environments (Stebbins 1985; Brochmann et al. 2004; Hijmans et al. 2007). In the case of *P. australis*, no such simple relationship is discernible, which is in line with the current view that the geographical and ecological distribution of polyploids follows no simple patterns (Otto and Whitton 2000; Husband et al. 2013). In *P. australis*, polyploidy was most prevalent in the southern populations located at lower elevations than in the northern sites we studied. However, except for increased temperature seasonality at the southern sites, mean annual temperatures are roughly comparable in the northern and southern parts of the range of *P. australis* (table 1; I. Hensen, unpublished data). Furthermore, the southernmost populations in the Sierras de Córdoba have a certain proportion of diploid trees, making it unlikely that the ploidy distribution in *P. australis* follows a simple latitudinal or environmental gradient. Interestingly, the high variation of ploidy levels in the Sierras de Córdoba contrasts with the pattern found in *Solanum* section *Petota*, in which the Andean species and populations have higher ploidy diversity (Hijmans et al. 2007). Unfortunately, we are unaware of other similar studies that may allow us to search for cross-taxon patterns in the distribution of ploidy levels in the Andes and nearby mountain ranges. In conclusion, we thus reject our hypothesis predicting increasing incidence of polyploidy at higher elevations, which is in accordance with a broader review by Hadač (1989).

More likely, the geographical distribution of ploidy types in *P. australis* reflects the area or areas of origin of polyploidy in the species and the consequent spread of this condition. Detailed molecular analyses will be necessary to unravel the evolutionary history, but a few conjectures can be drawn with the available evidence. Since the diploid state is ancestral in *Polylepis* (Schmidt-Lebuhn et al. 2010), the disjunct distribution of diploid plants may represent vicariant populations of the original stock. Under this scenario, tetraploids arose either once in the Andes or the Sierras de Córdoba, followed by long-distance dispersal between these areas, or separately in the two geographical areas. Because populations from the southern part of the Andean range portion of *P. australis* have previously been found to be genetically more similar to those in the Sierras de Córdoba than to more northern Andean populations (Hensen et al. 2011), the dispersal scenario appears to be the most plausible given current knowledge.

Finally, differences in ploidy level may have taxonomic implications. Events of both allo- and autopolyploidization are commonly considered to be linked with speciation events among plants (Soltis et al. 2007, 2009; Wood et al. 2009; Govindarajulu et al. 2012). In the Rosaceae, polyploidization has been linked to increased clade richness (Vamosi and Dickinson 2006) and to speciation events in, for example, the genera *Crataegus* (Lo et al. 2010) and *Sorbus* (Robertson et al. 2010; Pellicier et al. 2012). Interestingly, *P. australis* is among the morphologically most variable species of the genus (Bitter 1911; Simpson 1979; Schmidt-Lebuhn et al. 2006). Because ploidy levels in *Polylepis* can be inferred from stomatal measurements (Schmidt-Lebuhn et al. 2010), it may be possible to assess ploidy levels on herbarium material and to link morphological traits with ploidy levels. It is also currently unclear to which degree the triploid plants of *P. australis* are reproductively active because, despite detailed studies on the repro-

ductive ecology of the species (Seltmann et al. 2007, 2009), previous ignorance of the presence of polyploidy precludes relating any known variation in reproductive traits to ploidy levels. In other members of the Rosaceae, diploid-triploid-tetraploid networks are made possible by gametophytic apomixis (Dickinson et al. 2007), and it would be exciting to explore whether this is also the case in *P. australis* and congeners. However, the hard-shelled nutlets and the apparently short-lived endosperm of *Polylepis* seeds have so far hampered assessments of apomixis by flow cytometry (M. Kessler, personal observation).

Regardless of the underlying evolutionary process leading to the geographical distribution of ploidy levels in *P. australis*, our documentation of this pattern has important applied implications. Forests of *P. australis* are highly fragmented and the focus of conservation and restoration activities (Renison et al. 2002, 2011; Marcora et al. 2008). While the use of autochthonous material has already previously been advocated (Renison et al. 2005), our documentation of different ploidy levels further cautions against transporting seeds or plants between geographical areas, even in such a relatively constricted mountain range as the Sierras de Córdoba. For example, cultivation of diploid plants next to native tetraploids (and vice versa) might result in the production of sterile triploid offspring, potentially lowering the reproductive capacity of such populations. Similar thoughts apply to other species of *Polylepis*, especially those with extensive geographical ranges, marked morphological variability, and presumably also to variable ploidy such as *P. incana*, *P. pauta*, *P. racemosa*, and *P. sericea* (Simpson 1979; Schmidt-Lebuhn et al. 2006, 2010; Segovia et al. 2012).

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