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RESEARCH PAPER

An Andean radiation: polyploidy in the tree genus *Polylepis* (Rosaceae, Sanguisorbeae)

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ABSTRACT

The Andean tree genus *Polylepis* (Rosaceae) is notorious for the high morphological plasticity of its species and the difficulty in their circumscription. The evolutionary mechanisms that have driven diversification of the genus are still poorly understood, with factors as diverse as ecological specialisation, reticulate evolution, polyploidisation and apomixis being proposed to contribute. In the present study, chromosome counts, flow cytometry and stomata guard cell size measurements were employed to document for the first time the presence of polyploidy in the genus and to infer ploidy levels for most species. Inferred ploidy levels show a clear progression from diploidy in cloud forest species to polyploidy (tetra- to octoploidy) in the morphologically and ecologically specialised *incana* group, indicating that polyploidisation may have played a major role in speciation processes and the colonisation of novel habitats during the Andean uplift. At least two species of *Polylepis* comprise populations with varying degrees of ploidy. More extensive studies are needed to obtain a better understanding of the prevalence and effects of intraspecific polyploidy in the genus.

INTRODUCTION

The Andes are one of the global centres of plant diversity and endemism (Myers *et al.* 2000; Barthlott *et al.* 2007). The Andean uplift started about 20 Mya (Burnham & Graham 1999) but alpine habitats at and above the treeline are fairly young, dating from the Pliocene (Gregory-Wodzicki 2000). Yet these biomes are home to some of the most impressive plant radiations on Earth (Linder 2008), including *Lupinus* (81 spp., Hughes & Eastwood 2006), *Gentianella* (170 spp., von Hagen & Kadereit 2001), *Valeriana* (160 spp., Bell & Donoghue 2005), Ericaceae (800 spp., Luteyn 2002), and *Calceolaria* (230 spp., Molau 1988). The processes that lead to these radiations presumably include adaptive shifts to new high-elevation habitats as these became available during orogeny, isolation of organisms on different mountain ranges and slopes, and range shifts due to past climatic fluctuations (Hooghiemstra & Van der Hammen 2004; Brumfield & Edwards 2007). At the same time, because of their young age, many species in these radiations are rather ill defined, with hybridisation and introgression being commonly inferred from morphology. As such, the mechanisms leading both to the diversification of the taxa as well as to maintenance of species identities are of interest.

Polylepis (Rosaceae, Sanguisorbeae) is a genus of about 15–30 wind-pollinated tree and shrub species growing at high elevations in the Andes from Venezuela to Argentina (Bitter 1911; Simpson 1979; Kessler 1995a,c; Kessler & Schmidt-Lebuhn 2006). *Polylepis* is ecologically important

because it is the dominant and often only tree genus at elevations above *ca*. 3500 m in the Andes (Simpson 1986; Kessler 1995b). It is estimated that over 98% of the original forest cover has been lost due to human activities in the last few 1,000 year (Kessler 1995b, 2000; Fjeldså & Kessler 1996; Renison *et al.* 2006), rendering *Polylepis* forest among the most threatened ecosystems in the neotropics (Purcell *et al.* 2004).

Polylepis is most closely related to mainly southern temperate *Acaena* and presumably evolved during the uplift of the Andes in the Plio-Pleistocene (Simpson 1986; Eriksson *et al.* 2003; Kerr 2003). Within *Polylepis*, there is a well defined ecological and morphological gradient from tall trees with multiple, thin-textured leaflets and multiflowered inflorescences growing at the upper limits of humid cloud forests, to successively smaller species with reduced, coriaceous leaves and small inflorescences growing in higher and drier habitats (Simpson 1979, 1986; Kessler 1995b; Kerr 2003). This evolutionary progression, which parallels the development of major biomes in the Andes (Hooghiemstra & Van der Hammen 2004), is well supported by both morphological and molecular characters (Simpson 1979, 1986; Schmidt-Lebuhn *et al.* 2006a). Although the radiation of *Polylepis* is not quite as impressive as that of other high-Andean genera such as *Lupinus* and *Gentianella*, *Polylepis* is an ecologically dominant member of one of the world's richest high-mountain floras and an example of a plant genus that has rapidly evolved into new niches as these became available during the Andean uplift.

Ecological and biogeographical studies in *Polylepis* have long been hampered by problems in species delimitation. On the one hand, many taxa in *Polylepis* are morphologically quite similar, while on the other different geographical populations assigned to the same species are often diagnosably different, both morphologically and genetically (Simpson 1979, 1986; Schmidt-Lebuhn *et al.* 2006a). The causes of this variability are poorly understood, but presumably include hybridisation (Kessler 1995a,b; Romoleroux 1996; Kerr 2003; Schmidt-Lebuhn *et al.* 2006a,b, 2007) as well as the small size of the frequently disjunct relict populations of many species (Kessler 1995a; b). Polyploidisation has also been hypothesised to occur in *Polylepis* (Simpson 1979, 1986; Schmidt-Lebuhn *et al.* 2006a), because this phenomenon is widespread in disparate genera of the Rosaceae (Dickson *et al.* 1992; Dickinson *et al.* 2007) such as *Acaena* (Roulet 1981), *Alchemilla* (Gehrke *et al.* 2008), *Crataegus* (Talent & Dickinson 2005), and *Rosa* (Ritz *et al.* 2005). For example, in *Acaena* chromosome numbers of (41) 42, *ca.* 72, 84 and 126 have been documented (Zhukova 1967; Moore 1973, 1981; Roulet 1981; Beuzenberg & Hair 1983; Goldblatt 1985; Goldblatt & Johnson 1990). However, to date no precise chromosome counts are available for any species of *Polylepis*. Simpson (1979) mentioned that counts were attempted but failed, without giving specifics. Kessler (1995b) observed chromosome numbers of *ca*. 80 in root tips of three species of *Polylepis* (*Polylepis neglecta*, *Polylepis triacontandra*, *Polylepis tarapacana*), but exact counts were impossible due to the exceedingly small size of the chromosomes (*ca*. 1–2 μm) that led to confusion with dirt and stain particles.

Because the occurrence of polyploidisation may offer clues to better understand both the radiation of *Polylepis* and the species-level taxonomy, in the present study we carried out a concerted effort to document ploidy levels in the genus, taking advantage of a living collection of 11 species of *Polylepis* and using three complementary methods. We once again tried to conduct direct microscopic counts of chromosomes from root tips. As a second method, flow cytometry was used to measure nuclear genome sizes. Flow cytometric measurement of nuclear DNA content is a well-established technique that is now routinely used as a proxy for chromosome counts in ploidy level studies (*e.g.*, Tuna *et al.* 2001; Emshwiller 2002). Although considerable variation in the genome size of congeners of the same ploidy level has been reported for some genera (*e.g.*, Wendel *et al.* 2002), its use should be unproblematic provided that genome sizes in the group under study can be shown to fall into discrete batches, having the size ratios expected from polyploid series (Talent & Dickinson 2005). This should, presumably, often be the case for young groups consisting of very closely related species. Third, we measured guard cell lengths from both fresh and herbarium material. Guard cell size has repeatedly been shown to closely correlate with ploidy levels in a wide range of plant groups (*e.g.*, Masterson 1994; Sugimoto-Shirasu & Roberts 2003). Each of these three methods has its advantages and problems. Only direct counts provide specific chromosome numbers, whereas the other two approaches only allow indirect inferences. Furthermore, chromosome counts require living plant material, and for flow cytometric analysis fresh material is also recommended, although the first protocols that used dehydrated tissues have recently become available (Suda & Trávnícek 2006). However, living plant material of *Polylepis* is problematic to obtain because this genus is difficult to cultivate outside of tropical high-elevation conditions.

MATERIALS AND METHODS

Sampling

Living plant samples for flow cytometry were obtained from a collection of *Polylepis* species established at the Experimental Botanical Garden of Göttingen University, Germany. For each of the 11 species represented in the collection, two specimens were selected for the study (Table 1). In all species except *Polylepis australis*, all plants of a given species originated from the same wild populations. In addition, for the guard cell measurements we selected herbarium material of two specimens from each of the 27 species represented in the herbarium of the University of Göttingen (GOET) (out of the *ca*. 29 currently accepted species, Kessler & Schmidt-Lebuhn 2006)

Table 1. Mean 2C values measured for 22 specimens of 11 species of *Polylepis*. Voucher specimens are deposited at GOET.

species and voucher	specimen	provenance	replicates	genome size (pg)/2C (±SD)	mean for species
Polylepis australis Bitter (*Hertel 454*)	1	Old Botanical Garden of Göttingen, stock of unknown origin	3	2.977 (±0.060)	3.004 (±0.153)
	2	Grown at Botanical Garden of Halle, originally from Córdoba, Argentina	3	3.030 (±0.027)	
Polylepis hieronymi Pilger (*Hertel 453*)	1	Grown at Experimental Botanical Garden of Göttingen, originally from Tarija, Bolivia	3	1.487 (±0.042)	1.502 (±0.064)
	2	Grown at Experimental Botanical Garden of Göttingen, originally from Tarija, Bolivia	3	1.518 (±0.022)	
Polylepis microphylla (Wedd.) Bitter (*Hertel 458*)	1	Grown at Experimental Botanical Garden of Göttingen from seeds collected near Cuzco, Peru	3	1.529 (±0.055)	1.532 (±0.129)
	2	Grown at Experimental Botanical Garden of Göttingen from seeds collected near Cuzco, Peru	3	1.534 (±0.071)	
Polylepis neglecta M. Kessler (*Hertel 456*)	1	Grown at Experimental Botanical Garden of Göttingen, originally from Santa Cruz, Bolivia	3	1.549 (±0.093)	1.542 (±0.153)
	2	Grown at Experimental Botanical Garden of Göttingen, originally from Santa Cruz, Bolivia	3	1.535 (±0.057)	
Polylepis pauta Hieron. (*Hertel 455*)	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.565 (±0.105)	1.588 (±0.217)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.610 (±0.106)	
Polylepis pepei Simpson	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.603 (±0.068)	1.651 (±0.123)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.699 (±0.053)	
Polylepis racemosa R. & P. (*Hertel 451*)	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	5.717 (±0.147)	5.738 (±0.415)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	5.760 (±0.263)	
Polylepis sericea Wedd.	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.634 (±0.147)	1.651 (±0.305)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	1.667 (±0.146)	
Polylepis subsericans Macbride (*Hertel 452*)	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	3.122 (±0.183)	3.164 (±0.303)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Cuzco, Peru	3	3.205 (±0.112)	
Polylepis tarapacana Philippi (*Hertel 459*)	1	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Oruro, Bolivia	3	3.016 (±0.170)	3.010 (±0.338)
	2	Grown at Experimental Botanical Garden of Göttingen from seedlings collected in Oruro, Bolivia	2	3.004 (±0.159)	
Polylepis tomentella Wedd. ssp. *nana* M. Kessler (*Hertel 457*)	1	Grown at Experimental Botanical Garden of Göttingen, originally from Cochabamba, Bolivia	3	2.934 (±0.048)	2.949 (±0.096)
	2	Grown at Experimental Botanical Garden of Göttingen, originally from Cochabamba, Bolivia	3	2.964 (±0.047)	

in order to obtain full taxonomic coverage of the genus. Root tips of *Polylepis hieronymi*, *Polylepis neglecta*, *Polylepis racemosa* and *Polylepis tomentella* subsp. *nana* for chromosome counts were taken from the living collection at Göttingen.

Chromosome counts

Root tips (*ca*. 5–8 mm in length) were collected into 2-ml reaction tubes in the morning up to midday at different times of year. Roots were easily accessible because the plants are cultivated in pots. Four pre-fixation treatments of the tips were tested for their effectiveness in arresting mitosis and their effect on chromosome condensation: (i) with water for 4 h at *ca*. 0–4 °C; (ii) with *ca*. 2 mM 8-hydroxychinoline for 4 h at room temperature; (iii) with *ca*. 0.01% colchicine for 4 h at room temperature; and (iv) with *ca*. 1.25 mM 8-hydroxycuinoline and 0.05% colchicine for 4 h at room temperature. In the first three cases, the tips were then fixed overnight at room temperature in a mixture of ethanol:acetic acid (4:1). In the fourth case, the mixture also contained 2% polyvinylpyrrolidone (PVP), a substance that precipitates phenols. Before use, the tips were stored at −20 °C, for shorter periods in the ethanol–acetic acid mixture and for longer periods in 70% ethanol.

As a preparation for squashing, tips were washed three times for *ca*. 5 min in 0.01 M citric acid/sodium acetate buffer (pH 4.8), digested for *ca*. 40 min at 37 °C with a mixture of 1% cellulase and 10% pectinase, then washed again three times. Subsequently, each root tip was transferred onto a microscope slide and its root cap removed. A mixture of 2% carmine and propionic acid was added, and the root tip was macerated, covered with a cover slip and heated for *ca*. 3 s above a naked flame, then carefully squashed and examined under the microscope. In total, 72 root tips of the four selected taxa were examined, the majority belonging to *P. hieronymi* and *P. racemosa*.

Flow cytometry

For flow cytometric estimation of DNA content, approximately 1 cm^2 of young leaf material from a sample, together with an internal genome size standard, was chopped using a sharp razor blade in a Petri dish containing 1 ml chopping buffer according to Galbraith *et al.* (1983), supplemented with 5% (w/v) polyvinylpyrrolidone 25, 50 $\mu g \cdot ml^{-1}$ DNase-free RNase and 50 $\mu g \cdot ml^{-1}$ propidium iodide. The suspensions were filtered through a 35-μm mesh and subsequently measured on a FACStar^{PLUS} (BD Biosciences, San José, CA, USA) equipped with an argon ion laser INNOVA 90C (Coherent, Palo Alto, CA, USA). Usually 5,000–10,000 nuclei per sample were analysed and the absolute DNA amounts of the samples were calculated based on the values of the G1 peak means. We used *Raphanus sativus* L. cv. Voran (Genebank Gatersleben, accession number: RA 34) as internal standard. This has a genome size of 1.11 pg/2C, as determined in comparative measurements with *Arabidopsis thaliana* (L.) Heynh. 'Columbia' (0.32/2C, Bennett *et al.* 2003). Specimens were analysed three times, and mean values and standard deviations of the measurements calculated. One individual of *Polylepis tarapacana* was only measured twice due to scarcity of living plant material.

Guard cell measurements

For the guard cell measurements, we selected three well-developed leaflets of average size relative to all leaflets present on a given plant or herbarium specimen. On each leaflet, we selected a central part halfway between the midvein and the margin to measure the guard cells. In this area, we first carefully removed the hair cover with a scalpel and brush. In species with a wax layer on the leaflet surface, we soaked the leaflets for 1–4 h in acetone or isopropanol, then rinsed them in clear water, and finally dried samples between tissue paper. Once all covering substances had been eliminated from the leaves, we applied clear nail varnish that was allowed to dry for 4–12 h before removal. Application of the varnish was complicated by the fact that the stomata of many *Polylepis* species are small and deeply sunken in stomatal pits. Varnish types with high viscosity frequently did not reach into these pits, whereas thin, viscous varnishes were often elastic upon drying and difficult to remove from the leaflet surface without distortion, leading to correspondingly unreliable measurements. Because of the different properties of the various species of *Polylepis* (size and depth of the stomatal pits, surface structure of the epidermis, *etc*.), we experimented with varnishes of different viscosity for each species using dilution series with acetone until reliable imprints were obtained. Once these were considered adequate, we then measured the guard cell length on 10 stomata from each sample at 400× magnification under a light microscope, and averaged these measurements.

RESULTS

Chromosome counts

The great majority of samples did not yield usable results, partly due to the lack of metaphases in the cells, partly because metaphase plates consisted of chromosomes lumped so closely together that their discrimination was impossible. The most promising samples were those collected in the morning during summer and treated with hydroxychinoline solution. As in previous attempts by other researchers, the extremely small size of *Polylepis* chromosomes added to the difficulties encountered in cytological studies of the genus, rendering it virtually impossible to obtain exact counts. In total, only one sample of *Polylepis hieronymi* was of sufficient quality to be estimated at 2n = *ca*. 40 (Fig. 1a). A second sample, of

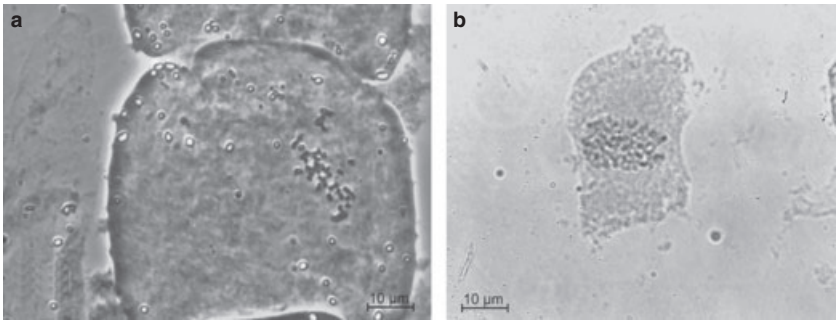

Fig. 1. Metaphases in *Polylepis hieronymi* (a) and *Polylepis racemosa* (b).

Polylepis racemosa, yielded metaphases with chromosomes lumped too densely together to allow for a similar confident estimate but appeared to exceed this number by a considerable margin (Fig. 1b).

Flow cytometry

The calculated absolute 2C values in *Polylepis* (Table 1) can be grouped into three clearly distinct, non-overlapping groups (Figs 2 and 3). The first included *P. hieronymi*, *Polylepis microphylla*, *Polylepis neglecta*, *Polylepis pauta*, *Polylepis pepei* and *Polylepis sericea*, with a minimum of 1.489 pg and a maximum of 1.699 pg. The second group included *Polylepis australis*, *Polylepis subsericans*, *Polylepis tarapacana* and *Polylepis tomentella* ssp. *nana*, and had a minimum of 2.924 pg and a maximum of 3.025 pg. One species, *P. racemosa*, showed an even higher 2C value, with an average of 5.737 (±0.065) pg, a minimum of 5.646 pg and a maximum of 5.830 pg. An additional species, *Polylepis besseri*, was submitted to preliminary analyses some months before the start of the systematic measurements presented here, when it was recorded with a 2C value about three times that of *P. hieronymi* (A. N. Schmidt-Lebuhn and J. Fuchs personal observation). Unfortunately, the cultivars of *P. besseri* did not survive into the following months, and could not be included in the current study for detailed investigations.

Guard cell measurements

Guard cell size varied from 10.2 to 20.4 µm between individual specimens (Table 2). The difference between conspecific samples measured with the same type of material

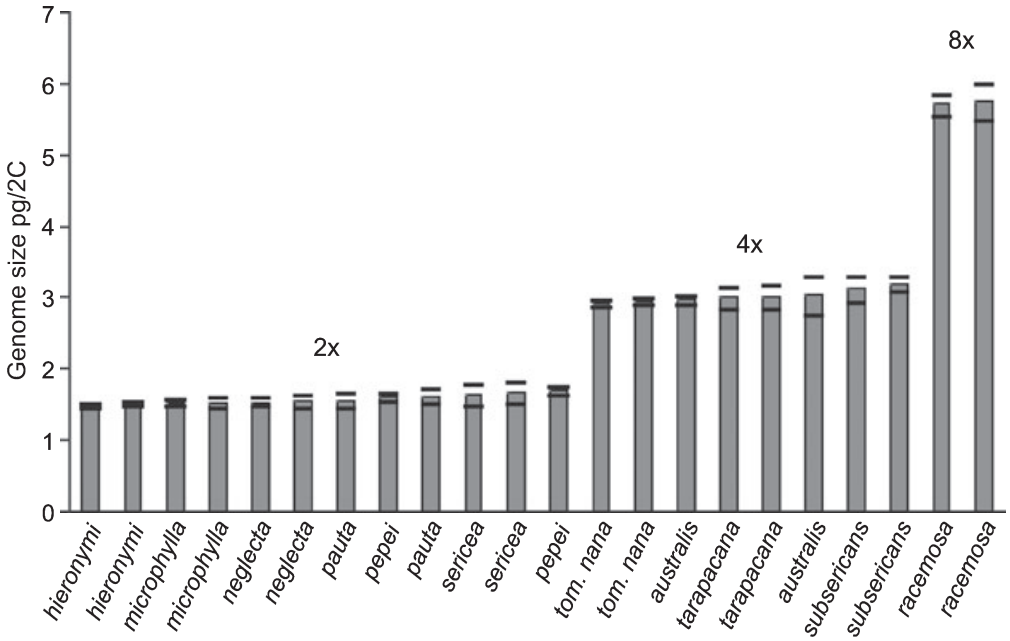

Fig. 2. Genome size ranges as obtained from flow cytometry for presumed diploid, tetraploid and octoploid samples of *Polylepis*. Samples are arranged from lowest to highest 2C value, independent of taxonomic affiliation. The dashes indicate standard deviations based on both repeat measurements and variability in size standard measurements.

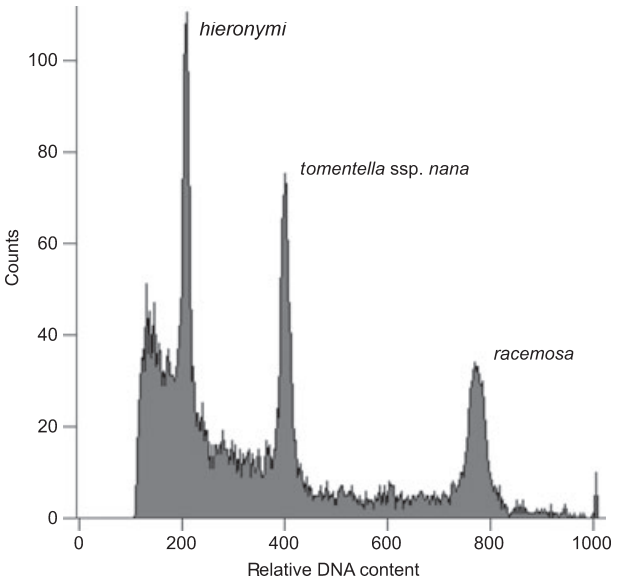

Fig. 3. Histogram of relative fluorescence intensities showing samples of a diploid, a tetraploid and an octoploid species of *Polylepis* analysed in the same preparation, without a size standard.

(living, herbarium) averaged 1.2 μm, which is much lower than the variation within the full dataset, showing that the measurements were repeatable and representative of the species. However, in three cases, the difference was more than 3 μm: one herbarium specimen of *P. australis* (*Lorentz 760*) had a mean length of 12.8 μm, whereas the other (*Kessler 3350*) had a mean length of 18.9 μm; in *P. pauta* one herbarium specimen (*Kessler 2749*) measured 12.5 μm and the other (*Laegaard 102327*) 16.5 μm; and in *P. racemosa* the two living specimens measured 16.7 and 20.2 μm.

For the 11 species for which we measured leaflets from both living and herbarium specimens, measurements between the two types of source material were in good agreement (Spearman rank correlation between mean values: $R = 0.93$, $P < 0.001$) (Fig. 4), suggesting that herbarium material can be used to compare guard cell length in *Polylepis*.

For the 11 living species included in the flow cytometry measurements, there was a significant difference in guard cell length of living specimens between species inferred to be diploid and tetraploid based on the 2C values (two-sample *t*-test, $t = -6.01$, $P < 0.001$) (Fig. 5). Likewise, a comparison between guard cell lengths of herbarium specimens of the same species was significant between the diploid and tetraploid species (two-sample *t*-test, $t = -5.31$, $P = 0.001$). In both living and dry material, all putative diploid species included in the flow cytometry analysis had mean guard cell lengths between 10.2 and 14.5 μm, whereas the putative tetraploid species all had measurements between 16.6 and 20.3 μm. The sole putative octoploid species included in the flow cytometry analysis, *P. racemosa*, had a mean guard cell length of

Table 2. Guard cell measurements of 74 specimens representing 27 taxa of *Polylepis*. For herbarium specimens, the collector and collection number are listed under 'source'. All herbarium specimens are deposited at GOET, live specimens are cultivated at the Experimental Botanical Garden of Göttingen University.

specimen	source	length (μm; mean ± SD)
Polylepis australis Bitter	live specimen 1	16.7 ± 2.6
	live specimen 2	17.4 ± 1.6
	Kessler 3350	18.9 ± 1.9
	Lorentz 760	12.8 ± 1.4
Polylepis besseri Hieron	*Kessler 2989*	20.4 ± 2.8
	Kessler 2985	19.2 ± 1.9
Polylepis crista-galli Bitter	*Beck 9343*	16.6 ± 1.4
	Kessler 3155	17.8 ± 2.1
Polylepis flavipila (Bitter)	*Kessler 3426*	18.0 ± 2.0
M. Kessler & Schmidt-Leb.	*Kessler 3591*	17.2 ± 2.2
Polylepis hieronymi Pilg.	live specimen 1	12.6 ± 1.8
	live specimen 2	11.9 ± 2.0
	Kessler 3123	11.2 ± 1.0
	Beck 9345	13.2 ± 1.8
Polylepis incana Kunth	*Schmidt-Lebuhn 521*	17.0 ± 2.3
	Laegaard 102647	17.6 ± 2.0
Polylepis incarum (Bitter)	*Jimenez 2716*	18.3 ± 0.8
M. Kessler & Schmidt-Leb.	*Kessler 3465*	17.2 ± 1.9
Polylepis lanata (Kuntze)	*Kessler 2851*	19.6 ± 2.1
M. Kessler & Schmidt-Leb.	*Kessler 2962*	18.8 ± 1.7
Polylepis lanuginosa Kunth	*Laegaard 102637*	11.8 ± 1.6
	Laegaard 55036	10.8 ± 1.4
Polylepis microphylla (Wedd.)	live specimen 1	13.9 ± 1.1
Bitter	live specimen 2	14.3 ± 2.2
	Galiano 1999	14.2 ± 1.8
Polylepis neglecta M. Kessler	live specimen 1	13.9 ± 1.1
	live specimen 2	14.3 ± 2.2
	Kessler 3531	13.6 ± 2.3
	Kessler 3633	13.2 ± 2.0
Polylepis pacensis	*Kessler 3028*	15.9 ± 2.7
M. Kessler & Schmidt-Leb.	*Mendez & Arcienaga 14*	17.7 ± 1.3
Polylepis pauta Hieron.	live specimen 1	10.6 ± 0.9
	live specimen 2	12.7 ± 1.8
	Kessler 2749	12.5 ± 1.8
	Laegard 102327	16.5 ± 2.8
Polylepis pepei B.B. Simpson	live specimen 1	10.2 ± 1.5
	live specimen 2	10.8 ± 1.3
	Kessler 2795	10.9 ± 1.6
	Kessler 3386	11.8 ± 1.6
Polylepis quadrijuga Bitter	*Gradstein s.n.*	12.2 ± 1.7
	Gradstein s.n.	12.3 ± 2.0
Polylepis racemosa Ruiz & Pav.	live specimen 1	20.2 ± 3.3
	live specimen 2	16.7 ± 3.3
	Ferreyra 12418	18.0 ± 1.6
	Rosales 04	19.5 ± 0.8
Polylepis reticulata Hieron.	*Laegaard 102691*	10.0 ± 0.9
	Kessler 2746a	12.2 ± 1.4
Polylepis rugulosa Bitter	*Ferreyra 2594*	16.8 ± 1.9
Polylepis sericea Wedd.	live specimen 1	12.4 ± 2.4
	live specimen 2	13.0 ± 1.7
	Kessler 2880	14.3 ± 2.6

Table 2. (*Continued*)

specimen	source	length (μm; mean ± SD)
Laegaard 55665	10.8 ± 1.2	
Polylepis subtusalbida (Bitter)	*Kessler 2999*	17.2 ± 1.7
M. Kessler & Schmidt-Leb.	*Kessler 3414*	17.2 ± 2.2
Polylepis subsericans J.F. Macbr.	live specimen 1	16.6 ± 2.2
	live specimen 2	17.3 ± 2.2
	Toivonen s.n.	18.3 ± 1.5
	Toivonen s.n.	18.5 ± 2.4
Polylepis tarapacana Phil.	live specimen 1	17.4 ± 1.8
	live specimen 2	16.9 ± 2.3
	Kessler 3599	17.4 ± 2.2
	Kumar 6	17.1 ± 1.1
Polylepis tomentella Wedd.	*Kessler 3288*	16.4 ± 1.6
subsp. *incanoides* M. Kessler	*Kessler 3293*	18.3 ± 2.4
Polylepis tomentella Wedd.	live specimen 1	18.7 ± 1.7
subsp. *nana* M. Kessler	live specimen 2	19.6 ± 1.6
	Kessler 3514	20.3 ± 2.6
	Kessler 3642	19.5 ± 1.3
Polylepis tomentella Wedd.	*Kessler 3368*	18.7 ± 2.0
subsp. *tomentella*	*Kessler 3188*	17.9 ± 1.5
Polylepis triacontandra Bitter	*Kessler 3420*	20.4 ± 1.1
	Beck 4976	18.9 ± 1.9
Polylepis weberbaueri Pilg.	*Acleto 364*	12.3 ± 0.9
	Laegaard 102677	12.2 ± 1.0

17.5 μm and was therefore not distinguishable from the tetraploid species. Based on these values, we were able to assign the following species studied only as herbarium material as diploids: *Polylepis lanuginosa* (mean guard cell length 11.3 μm), *Polylepis quadrijuga* (12.2 μm), *Polylepis reticulata* (11.1 μm) and *Polylepis weberbaueri* (12.3 μm). Conversely, the following taxa are considered to be at least tetraploid based on guard cell measurements of herbarium material: *P. besseri* (19.8 μm), *Polylepis crista-galli* (17.2 μm), *Polylepis flavipila* (17.6 μm), *Polylepis incana* (17.3 μm), *Polylepis incarum* (17.7 μm), *Polylepis lanata* (19.2 μm), *Polylepis pacensis* (16.8 μm), *Polylepis rugulosa* (16.8 μm), *Polylepis subtusalbida* (17.2 μm), *P. tomentella* subsp. *incanoides* (17.3 μm), *P. tomentella* subsp. *tomentella* (18.3 μm) and *Polylepis triacontandra* (19.6 μm). The only problematic specimens in this assignation were those of *P. australis* and *P. pauta*, in which one specimen of each species had measurements corresponding to the diploid class, and the other to the polyploid class.

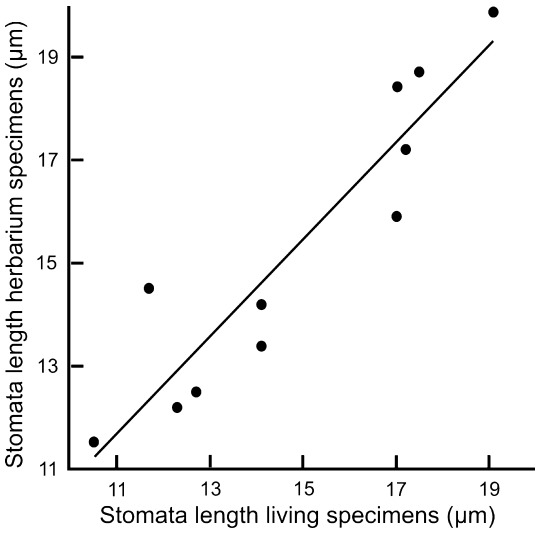

Fig. 4. Relationships in stomatal guard cell measurements of conspecific living and herbarium specimens.

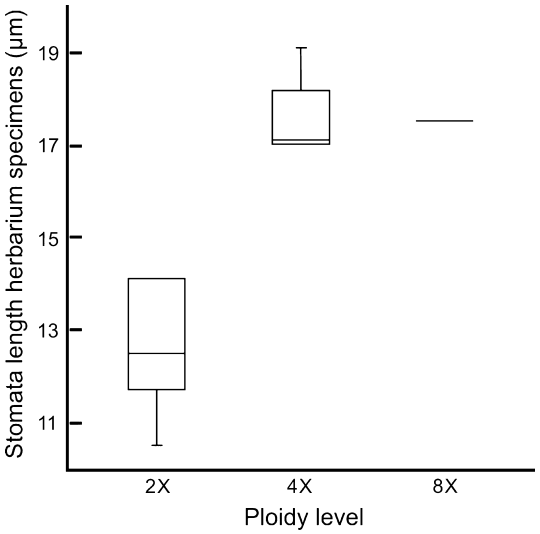

Fig. 5. Box plot diagram showing the variation in means of guard cell lengths for six diploid, four tetraploid and one octoploid species of *Polylepis*.

DISCUSSION

Inference of polyploidy in *Polylepis*

Calculated absolute 2C values obtained from flow cytometry can be grouped into three distinct groups whose mean values (1.572 ± 0.061, 3.007 ± 0.071 and 5.737 ± 0.065) stand in a proportion of 1.000:1.913:3.650, *i.e.*, *ca.* 1:2:4 to each other (Figs 2 and 3). This relationship and the very limited variability in the first two groups (Fig. 2) suggest that the three groups correspond to diploid, tetraploid and octoploid states, respectively. The presumably tetraploid and octoploid plant species do not have 2C values exactly twice or four times as high as the presumably diploid species. However, higher ploidy levels frequently do not exhibit the exact multiple DNA content that could be expected, but slightly less, presumably due to reorganisation of the genome and reduction of redundant information (Leitch & Bennett 2004). In addition, a certain deviation has to be expected as species used to estimate means are not necessarily phylogenetically independent

and, moreover, most likely underwent some independent genome modification during their individual speciation.

Guard cell measurements likewise revealed consistent patterns, with non-overlapping values between species inferred to be diploid and tetraploid in the flow cytometry measurements. Interestingly, the only octoploid species, *Polylepis racemosa*, did not differ in guard cell size from the tetraploid species, but the low sample size prevents interpretation of this result.

Unfortunately, direct chromosome counts were, once again, found to be almost impossible to achieve in *Polylepis*, the difficulty in proper fixation, as well as extremely small size and relatively high number making this plant genus one of the most challenging in this regard (G. Winterfeld personal communication). Nevertheless, the few borderline useable samples and previous observations appear to support the notion that the genus does not only show DNA polyploidy, but true chromosomal polyploidy (as defined by Suda *et al.* 2006): *Polylepis hieronymi* can be estimated at 2n = *ca.* 40 (Fig. 1A), which is in accordance with the results of the other two approaches used in this study, indicating this species to be diploid. Previous attempts at counting by Kessler (1995b) yielded estimates of 2n = *ca.* 80 for three other species, two of which are classified as polyploids in the guard cell measurements. In addition, *P. racemosa*, the only species showing DNA octoploidy in flow cytometry, appears to have a significantly higher number of chromosomes than *P. hieronymi*, even though confident estimates as to the precise number are impossible (Fig. 1B).

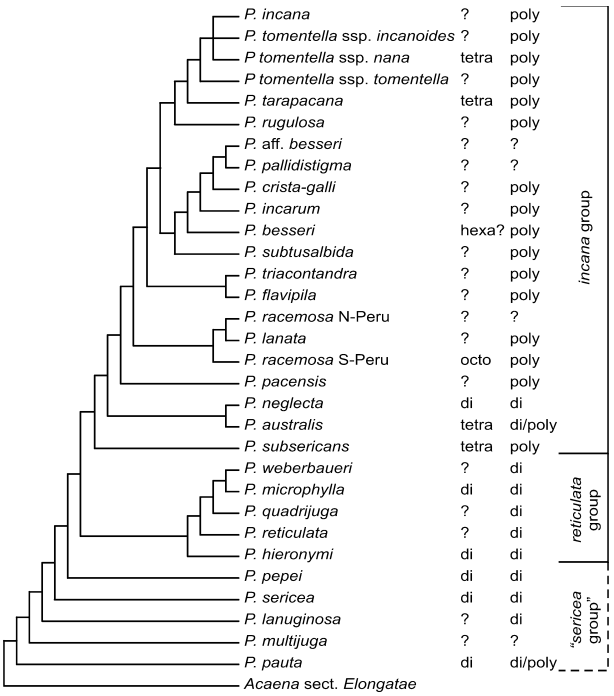

Fig. 6. Ploidy levels as inferred from calculated 2C values (left column) and guard cell measurements (right column) mapped onto a morphology-based cladogram adapted from Schmidt-Lebuhn *et al.* (2006a). Hexaploidy in *Polylepis besseri* is based on preliminary genome size measurements that could not be corroborated due to death of the cultivars.

Polyploidy in the diversification of *Polylepis*

The flow cytometry and guard cell measurements allow us to infer diploidy or polyploidy for most species of *Polylepis*. When these results are plotted on the phylogeny of *Polylepis* as inferred from morphological data in Schmidt-Lebuhn *et al.* (2006a), a very clear pattern is evident, with a progression from diploidy to higher ploidy levels, and almost all polyploid species restricted to the derived *incana* group (Fig. 6).

In this regard, *Polylepis* thus shows similar behaviour to other genera of Rosaceae that are well known for their polyploidy-driven diversification (*e.g.*, Dickson *et al.* 1992; Talent & Dickinson 2005; Dickinson *et al.* 2007). For some genera, the mechanisms behind polyploidisation and correlated speciation events have already been elucidated in great detail (Comai 2005; Talent & Dickinson 2007), and it would be interesting to learn how similar the case of *Polylepis* is. Combining nucleotide and chloroplast sequence data, Kerr (2003) inferred that *Polylepis* was derived from species of *Acaena* through multiple hybridisation events, which might have involved polyploidisation events. Consequently, it cannot be ruled out that the species discussed as diploid in this study are already ancient polyploids, but behave as functional diploids if the perspective is limited to *Polylepis* alone.

Another parallel can be drawn not to related taxa, but to speciation processes undergone by unrelated groups in similar habitats and under similar ecological pressures. *Polylepis* presumably diversified during the Andean uplift, colonising novel and increasingly alpine habitats in the process (Simpson 1986; Eriksson *et al.* 2003; Kerr 2003). In arctic and alpine habitats, polyploid species are especially numerous. For the Arctic, it has previously been argued that the basis of the success of polyploids lies in their enhanced ability to cope with inbreeding and genetic drift in periods of dramatic climatic change (and thus, dramatic contractions and expansions of their habitat) (Brochmann *et al.* 2004). The same case could be made for alpine habitats of the high Andes. For another young mountain range, Chen *et al.* (2007) recently demonstrated a correlation of polyploidy in Asian *Buddleja* with the Himalayan uplift, a result that shows obvious parallels to the situation apparently found in *Polylepis*. In a similar example for South America, Hijmans *et al.* (2007) observed that higher-level polyploids in *Solanum* section *Petota* tend to occur in colder areas. Interestingly, *P. racemosa*, the species with the highest ploidy level in our study, is well known in Peru as the fastest growing species of *Polylepis* (Pretell *et al.* 1985), and is widely cultivated outside of its native range (Simpson 1979; Fjeldså & Kessler 1996). Similarly, the closely related polyploid *P. lanata*

was found to grow into the largest tree individuals (>30 m in height, >150 cm in dbh) among all *Polylepis* species in Central Bolivia (Hertel & Wesche 2008). Hence, one could speculate that this species, which is most closely related to *P. racemosa*, may also show higher-level ploidy. It should be noted, however, that some species of the genus could be of hybrid and/or allopolyploid origin, so that interpretations based on our current inference of its phylogeny (Fig. 6) have to be treated with reservations.

Intraspecific variability

Besides the general patterns of a progression of ploidy in *Polylepis*, there are also two examples of mixed ploidy in single species or species pairs. The sister species *Polylepis australis* and *Polylepis neglecta* were inferred to be tetraploid and diploid, respectively, in the flow cytometry measurements. To further complicate the situation, the two studied herbarium specimens of *P. australis* were also assigned to different ploidy levels, and attempted chromosome counts in Kessler (1995b) indicated 2n = *ca*. 80 for *P. neglecta*. Apparently, in this species group, both diploid and tetraploid individuals are present. Whether this corresponds to geographical, ecological or morphological differences remains to be studied. It is noteworthy, however, that *P. australis* is one of the morphologically most variable species in the genus (Simpson 1979), with Bitter (1911) recognising 12 subspecies and varieties. It should also be mentioned that *P. australis* and *P. neglecta* occupy a phylogenetically isolated position in the mostly polyploid *incana* group, representing one of the deepest splits in the clade.

The other case of inferred mixed ploidy within a species is presented by *Polylepis pauta*. This is more surprising considering the phylogenetic position of the species among otherwise purely diploid species. *P. pauta* is, however, also a geographically very variable species (Simpson 1979), and the two herbarium samples studied by us are from Bolivia and Ecuador. It is thus conceivable that different ploidy levels occur in different populations of the species. Also, this would imply that polyploidy has arisen at several times independently in *Polylepis*. Alternatively, the assumed tetraploid sample of *P. pauta* could be allopolyploid and the result of hybridisation. Hybridisation is rampant within *Polylepis*, even involving distantly related species from different species groups (Kessler 1995a,b; Romoleroux 1996; Kerr 2003; Schmidt-Lebuhn *et al.* 2006a,b, 2007). In Ecuador, Romoleroux (1996) described putative hybrids between *P. pauta* and *P. incana* based on morphological characters. The latter is a tetraploid, which might explain the occurrence of tetraploid *P. pauta* in Ecuador.

CONCLUSIONS

Our study has, for the first time, confirmed the occurrence of polyploidy in *Polylepis* and has documented phylogenetic transitions between diploidy and polyploidy. There are, however, a number of unresolved issues. First, while most of the *incana* group can be considered to be polyploid, the exact ploidy levels are still unknown for those species not included in the flow cytometry analysis, because the guard cell sizes did not differ between the tetraploid and octoploid species. The confirmed presence of octoploidy in *Polylepis racemosa*, and the possible occurrence of hexaploidy in *Polylepis besseri* show that the distribution of ploidy levels in the *incana* group is complex. Second, for at least two species our survey indicates the occurrence of different ploidy levels within the same species. Given that we studied at most four individuals of any given species, it is conceivable that further cases of multiple within-species ploidy levels remain undetected. The extent of such cases, and their causes might allow insights into the mechanisms of diversification within *Polylepis*. Third, this raises – once again – the question of species delimitation in *Polylepis*. Soltis *et al.* (2007) argued that populations of different ploidy levels within single 'species' should be recognised as different species, in which case *Polylepis* might include more species than currently recognised. To complicate matters further, hybridisation in *Polylepis*, even across ploidy levels, may have resulted in closer genomic similarity between geographically close populations of different species than between geographically distant populations of the same species (Schmidt-Lebuhn *et al.* 2006a). If true, this calls for a genic view of speciation and species delimitation (Wu 2001), in which few genes are responsible for species separation, while the species boundary is simultaneously porous for much of the remainder of the genome. *Polylepis* might be a good example in which to study how adaptation to extreme environments, in combination with polyploidy, has resulted in selection for specific genes determining species limits.

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