Characterization of reproductive and cytological features of midday flowers (Aizoaceae) for breeding purposes

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Abstract

The succulent plant family Aizoaceae (midday flowers), whose members are mainly distributed in southern Africa, offers an enormous diversity of growth forms and flower pigmentations. Moreover, most species are drought tolerant. Despite their high ornamental value, breeding of midday flowers is still in its infancy and only few protected cultivars have been launched onto the market so far. Most likely, this fact can be ascribed to the presence of cross-incompatibilities and to a lack of knowledge on cytological properties and taxonomic relationships, which could facilitate the overcoming of hybridization barriers. These include genome sizes, chromosome numbers and knowledge on physiological mechanisms behind the cross-incompatibilities. Moreover, only little is known about flower-inducing factors and their control during midday flower cultivation. The performed analyses aimed at clarifying these relevant parameters.

Genotypes of the genera *Cephalophyllum*, *Lampranthus* and *Delosperma* were analyzed with respect to flower formation. Obligate photoperiodic responses were detected in none of the genotypes. The investigated *Lampranthus* genotypes formed significantly more flowers at mean daily temperatures of 14 °C than at 20 °C. Conversely, *Delosperma* tended to form more flowers at 20 °C. Flowering in the investigated *Cephalophyllum* genotype was not influenced by the chosen temperature regimes.

Intra- and intergeneric cross-pollinations were performed in *Lampranthus* and *Delosperma*. Even after intergeneric hybridizations, prezygotic barriers were not observed before pollen tubes entered the micropyles of ovules. More often, hybridizations failed due to postzygotic barriers. These became manifest in aberrant early embryogenesis, albinism and/or low hybrid vigor. However, some viable intra- and intergeneric hybrids were obtained by sterile sowings of seeds *in vitro* and employing the embryo rescue technique. The hybrid state of these plants was successfully confirmed by AFLP markers.

Various methods were applied to verify the presence of unreduced male gametes because pollen grains shed by the investigated *Delosperma* and *Lampranthus* genotypes distinctly varied in diameters and in sizes of containing sperm nuclei. Microscopic analyses of microsporogenesis revealed no aberrations from normal meiosis, and pollen grains could not be classified into different ploidy levels on the basis of their diameters. Flow cytometric analyses of pollen nuclei turned out to be inapplicable because pairs of sperm nuclei and intact male germ units (MGUs) caused the detection of fluorescence signals corresponding to DNA contents of 2C and 3C, which possibly overlapped the signals of unreduced gametes.

Genome sizes, however, were successfully estimated by flow cytometric measurements of pollen nuclei together with internal calibration standards. In *Delosperma*, these ranged from 1.18 pg/2C to 3.68 pg/2C and from 1.6 pg/2C to 2.36 pg/2C in *Lampranthus*.

Characterization of endoreduplication by flow cytometry revealed that tissues of most plant organs consisted of cells with up to five different DNA contents (2C-32C). The highest rates of endoreduplicated cells were detected in cotyledons (74-87 %), petaloid staminodes (56-95 %) and fully developed leaves (64-90 %), whereas relatively low portions were found in roots (23-34 %), internodes (29-45 %) and young leaves (17-56 %). Particularly organs that contain high proportions of 2C nuclei might be well-suited for shoot regeneration *in vitro*, as endoreduplicated cells are assumed to lose their ability to divide mitotically.

Keywords: cytological analyses, flower induction, hybridization barriers

Zusammenfassung

Die Familie der sukkulenten Mittagsblumengewächse (Aizoaceae), deren Mitglieder hauptsächlich im südlichen Afrika verbreitet sind, weist eine bemerkenswerte Vielfalt an Wuchsformen und Blütenfarben auf. Zudem besitzen die meisten Arten eine relativ hohe Toleranz gegenüber Trockenheit. Trotz ihres hohen Zierwertes ist die züchterische Bearbeitung von Mittagsblumengewächsen noch nicht weit fortgeschritten und derzeitig werden nur sehr wenige geschützte Sorten zum Verkauf angeboten. Die wahrscheinlichsten Gründe hierfür sind mangelnde Kreuzungskompatibilität, fehlende Informationen zu Verwandtschaftsverhältnissen sowie ein Mangel an zytologischen Daten, die die Überwindung von Kreuzungsbarrieren erleichtern könnten. Hierzu zählen unter anderem Genomgrößen, Chromosomenzahlen und Kenntnisse über die physiologischen Hintergründe von Inkompatibilitäten. Außerdem ist nur wenig über blühinduzierende Faktoren, und wie diese bei der Kultivierung von Mittagsblumengewächsen genutzt werden können, bekannt. Die dargestellten Untersuchungen wurden mit dem Ziel durchgeführt, möglichst viele dieser züchtungsrelevanten Parameter aufzuklären.

Genotypen der Gattungen *Cephalophyllum*, *Lampranthus* und *Delosperma* wurden hinsichtlich ihrer Blühreaktionen untersucht. Ein obligater photoperiodischer Einfluss auf die Blühinduktion konnte in keinem Fall festgestellt werden. Während die untersuchten *Lampranthus*-Genotypen bei einer Tagesmitteltemperatur von 14 °C deutlich mehr Blüten ausbildeten als bei 20 °C, wurde die Blütenbildung bei *Delosperma* durch höhere Temperaturen gefördert. Bei dem untersuchten *Cephalophyllum*-Genotyp wurde kein Einfluss der Tagesmitteltemperatur festgestellt.

Kreuzungsversuche wurden innerhalb und zwischen den Gattungen *Lampranthus* und *Delosperma* durchgeführt. Präzygotische Hybridisierungsbarrieren traten selbst in intergenerischen Kreuzungen erst nach Eintritt von Pollenschläuchen in die Mikropylen von Samenanlagen auf. Weitaus häufiger wurden postzygotische Kreuzungsbarrieren festgestellt, die sich vor allem in anormaler Entwicklung junger Embryonen und in partiellem oder vollständigem Fehlen von Chlorophyll bzw. geringer Vitalität der Nachkommenschaft äußerten. Dennoch gelang durch In-vitro-Aussaat und die Anwendung der Embryo Rescue-Methode die Erzeugung einiger lebensfähiger intra- und intergenerischer Hybriden, deren Hybridstatus nachfolgend durch AFLP-Marker verifiziert werden konnte.

Da die untersuchten *Lampranthus*- und *Delosperma*-Genotypen Pollenkörner freisetzten, die sich hinsichtlich ihres Durchmessers und der Größe der in ihnen enthaltenen Spermakerne stark unterschieden, wurden unterschiedliche Methoden zum Nachweis unreduzierter männlicher Gameten eingesetzt. Mikroskopische Untersuchungen der Mikrosporogenese ergaben keinerlei Hinweise auf meiotische Abweichungen, und Pollenkörner konnten nicht allein anhand ihrer Größe unterschiedlichen Ploidiestufen zugeordnet werden. Durchflusscytometrische Analysen von Pollenkernen erwiesen sich als ungeeignet, da gepaarte Spermakerne und vollständige „male germ units“ (MGUs) zur Detektion von Fluoreszenzsignalen führten, die 2C und 3C DNA-Gehalten entsprachen und dadurch den Detektionsbereich unreduzierter Gameten überlagerten.

Genomgrößen konnten hingegen durchflusscytometrisch durch gemeinsame Messung von Pollenkernen und internen Größenstandards ermittelt werden. Bei *Delosperma* lagen diese zwischen 1,18 pg/2C und 3,68 pg/2C und bei *Lampranthus* zwischen 1,6 pg/2C und 2,36 pg/2C.

Durchflusscytometrische Untersuchungen zur Charakterisierung der Endoreduplikation ergaben, dass die Gewebe fast aller Pflanzenorgane aus Zellen mit mindestens fünf unterschiedlichen DNA-Gehalten (2C-32C) bestanden. Besonders hohe Anteile endoreplizierter Zellen wurden in Kotyledonen (74-87 %), petaloiden Staminodien (56-95 %) und vollständig entwickelten Blättern (64-90 %) nachgewiesen, während sie in Wurzeln (23-34 %), Internodien (29-45 %) und jungen Blättern (17-56 %) vergleichsweise wenig vertreten waren. Insbesondere Organe mit einem hohen Anteil von 2C-Zellen könnten für Sprossregenerationen *in vitro* geeignet sein, da von endoreplizierenden Zellen angenommen wird, dass sie sich nicht mehr teilen können.

Schlagworte: Blühinduktion, Hybridisierungsbarrieren, Zytologische Untersuchungen

Publications derived from this thesis

Research papers

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Conference contributions

Talks

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Posters

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Braun P., Winkelmann T. (2014) Anwendung der Differentialinterferenzkontrast (DIC)-Mikroskopie zur Identifizierung von Hybridisierungsbarrieren zwischen Mittagsblumengewächsen (Aizoaceae). 49. Gartenbauwissenschaftliche Jahrestagung der DGG und des BHGL. Dresden, Germany; 05-08/03/2014

Braun P., Winkelmann T. (2015) Charakterisierung der Endoreduplikation bei Mittagsblumengewächsen (Aizoaceae). 50. Gartenbauwissenschaftliche Jahrestagung der DGG und des BHGL. Weihenstephan, Germany; 24-28/02/2015

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Abbreviations

α	significance level
µm	micrometer
(v/v)	volume/volume
(w/v)	weight/volume
x g	times gravity
AFLP	amplified fragment length polymorphism
C	"constant"; amount of DNA contained within a haploid nucleus
CAM	crassulacean acid metabolism
CO	CONSTANS
CDK	cyclin-dependent kinase
cp	cytoplasmic projection
CRP	cysteine-rich protein
CTAB	cetrimonium bromide
CV	coefficient of variation
CYC	cyclin
D.	*Delosperma*
DAP	days after pollination
DAPI	4',6-diamidino-2-phenylindole
DIC	differential interference contrast
DNA	deoxyribonucleic acid
e.g.	*exampli gratia* (lat.: for example)
em	embryo
es	embryo sac
et al.	*Et alii* (and others)
EtOH	ethanol
FDA	fluorescein diacetate
FDR	first division restitution
FLC	*FLOWERING LOCUS C*
fn	funiculus
FT	*FLOWERING LOCUS T*
GA	gibberellic acid
GABA	γ-aminobutyric acid
GCFR	Greater Cape Floristic Region (*sensu* Born et al., 2007)
gcw	generative cell wall
GISH	genomic *in situ* hybridization
h	hours
H_2O	water
HCl	hydrochloric acid
K_2O	potassium oxide
$K_3PO_4H_2O$	tri-potassium phosphate monohydrate
klx	kilolux
KOH	potassium hydroxide
L.	*Lampranthus*
LDP	long-day plant
LED	light-emitting diode
LSI	late-acting self-incompatibility
M	molar
MDT	mean daily temperature
MGU	male germ unit
mm	millimeter
mp	micropyle
MS	Murashige & Skoog
n	gametic chromosome number

N	nitrogen
N_2O	nitrous oxide; laughing gas
Na_2EDTA	ethylenediaminetetraacetic acid disodium
NaCl	sodium chloride
nm	nanometer
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NI	night interruption
no.	number
ov	ovule
P_2O_5	phosphorus pentoxide
PCR	polymerase chain reaction
pg	picogram
pla	placenta
PPFD	photosynthetic photon flux density
pt	pollen tube
PVP	polyvinylpyrrolidone
RNAse	ribonuclease
SD	standard deviation
SDP	short-day plant
SDR	second division restitution
SI	self-incompatibility
sn	sperm nucleus
sp	suspensor
st	stigma
TE	tris-EDTA
TEs	transposable elements
Tris	tris(hydroxymethyl)aminomethane
tt	transmitting tract
vn	vegetative nucleus
WAP	weeks after pollination
WGD	whole-genome duplication
zy	zygote

1. General Introduction

1.1 Phylogenetic background of the investigated Aizoaceae genera

The Aizoaceae belongs to the clade core eudicots and the order Caryophyllales (APG III, 2009). Taxonomic classifications within the family, which for a long time based on morphological characteristics (e.g. Ihlenfeldt, 1960; Haas, 1976; Bittrich and Hartmann, 1988; Chesselet et al., 2002) and in recent years primarily on molecular analyses (e.g. Klak et al. 2003, 2007, 2013; Thiede 2004) were often discussed controversially and revised several times. In this way, a monogeneric group of plants (*Mesembryanthemum*) that was described by Linnaei (1753) turned into the most species-rich family of succulent plants worldwide (Klak et al. 2003). At present, the Aizoaceae covers approximately 1800 species in 135 genera, which split up in the four subfamilies Sesuvioideae, Aizoideae, Mesembryantemoideae and Ruschioideae (Klak et al., 2003; Thiede, 2004).

The genotypes investigated in the present thesis belong to the Ruschioideae genera *Cephalophyllum*, *Delosperma* and *Lampranthus* and were provided by the ornamental plant breeding company Selecta One. Unfortunately, detailed information about their origin or taxonomic background at species level were not available for most genotypes. The Ruschioideae is by far the largest of the four subfamilies. It covers approximately 1585 species in 111 genera and is subdivided in the three tribes Apatesieae, Dorotheantheae and Ruschieae. The genera above are part of the Ruschieae tribe, which exclusively consists of succulent perennials (Chesselet et al., 2002). Interestingly, the Ruschieae tribe (also referred to as `core ruschioids´) was identified as the most rapidly diversifying known group of plants (Klak et al., 2004) and most speciation events date back less than four million years (Valente et al., 2014).

Relatively few chromosome counts have been published for the Aizoaceae. However, all studied Ruschioideae taxa were found to have a diploid chromosome number of 2n=18, but also tetraploid and hexaploid specimens were detected within species boundaries (Wulff, 1944; Albers and Haas, 1978; Hartmann, 1986; Hammer and Liede 1992). The genome sizes of Aizoaceae species were assumed to be generally small (< 1.4 pg/1C; Soltis et al. 2003), but so far genome size data has been only available for the Mesembryanthemoideae species *Mesembryanthemum crystallinum* (approx. 0.9 pg/2C; De Rocher et al., 1990; Bennett and Leitch, 1995).

1.2 Morphology and distribution

1.2.1 General remarks

Aizoaceae taxa occur in all vegetation types of southern Africa (Fig. 1), but the vast majority of species is endemic to winter rainfall regions located nearby the South African Cape (Van Jaarsveld and De Pienaar, 2004), also referred to as Greater Cape Floristic Region (GCFR; Born et al., 2007). Relatively few genera occur outside of Africa, e.g. in North and South America, Arabia, Australia, Madagascar, New Zealand and around the Mediterranean Sea (Smith et al., 1998). In the family´s area of origin, important diversity hotspots are located in the Succulent Karoo, a semi-arid biome that comprises coastal plains and intermontane valleys lying along the western and southern edges of the Great Escarpment (Milton et al., 1997). The growing season of taxa in this core area takes place at rather moderate temperatures during winter, while the dry, hot summer months are survived in a resting state. Due to their individual habitat requirements, many species are extremely limited in their distribution and threathened with extinction (Smith et al., 1998). In respect of their growth type and

degree of succulence, members of the Aizoaceae family can be classified in three different groups: (1) herbaceous, ephemeral plants with low succulence, (2) shrubby, perennial plants with medium succulence and (3) perennial, extremely succulent plants (Ihlenfeldt, 1960). Irrespective of the subfamily, Aizoaceae species were repeatedly found to have the ability to switch their mode of photosynthesis from C_3 to CAM (Crassulacean acid metabolism; Winter 1973; Adams et al., 1998; Matimati et al., 2012). Ruschioideae taxa generally form actinomorphic flowers with inferior ovaries (Ihlenfeldt, 1960; Volgin, 1998). Due to an alternative flower organ identity program, the intensively colored flower leaves are petaloid staminodes (Brockington et al., 2012). The diurnal movement of petaloid staminodes in the Aizoaceae (hence the name `midday flowers´) is mainly correlated with temperature and is assumed to protect pollen from exposure to moisture (von Hase et al., 2006). According to Smith et al. (1998), pollination is generally performed by insects. As in other Caryophyllales families, the flower leaves of Aizoaceae taxa contain betalains, which are responsible for their unique pigmentations (Brockington et al., 2011). According to extensive investigations on flower development conducted by Ihlenfeldt (1960), protandry seems to be widespread within the family. Most midday flowers form dry, hydrochastic seed capsules, which open after moistening and close again when dry. The seeds within are dispersed by raindrops (Ihlenfeldt, 1960; Van Jaarsveld and De Pienaar, 2004).

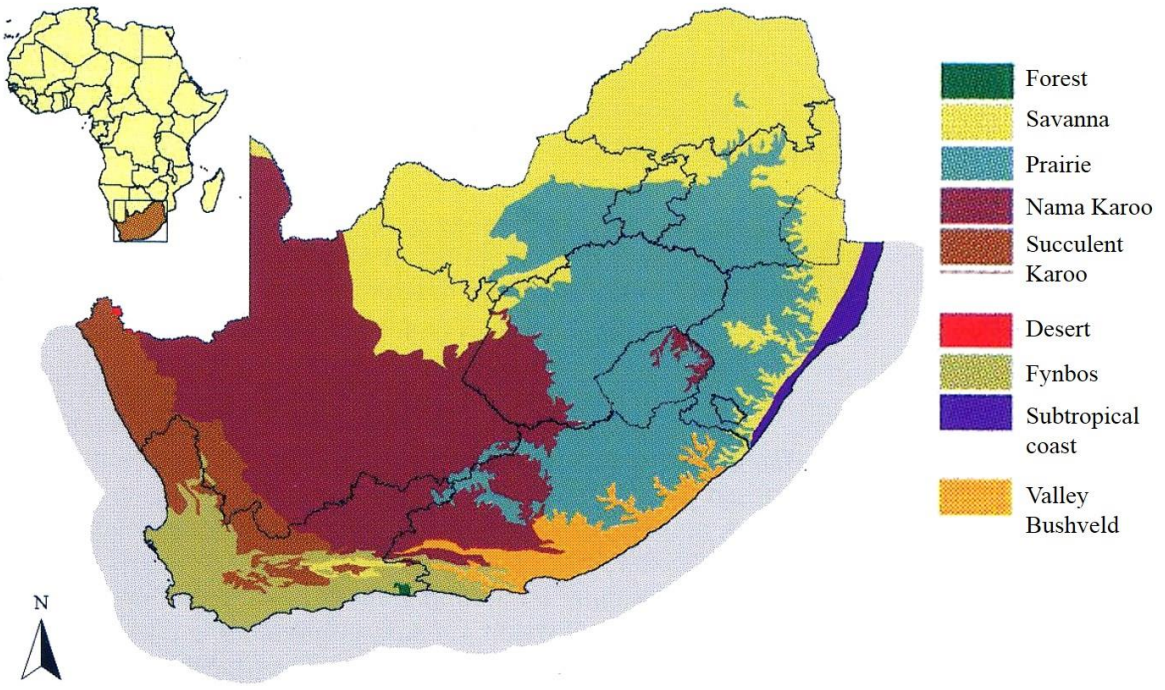

Fig. 1 Vegetation types of southern Africa (Van Jaarsveld and De Pienaar, 2004, modified)

1.2.2 *Cephalophyllum*

The genus *Cephalophyllum* is subdivided in the two subgenera *Cephalophyllum* and *Homophyllum*, which can be distinguished by morphological characteristics (Hartmann, 2001). While in members of the subgenus *Cephalophyllum* the primary stem forms a central head of leaves (Greek: cephalos = head) (Hartmann, 2001), plants of the subgenus *Homophyllum* are characterized by leaves of equal size (Smith et al., 1998). Taken as a whole, the genus *Cephalophyllum* covers approximately 33 (Smith et al., 1998) to 38 species (Van Jaarsveld and De Pienaar, 2004), which exclusively occur in winter rainfall regions of the northwestern and western Cape of South Africa with less than 400 mm annual precipitation. The plants grow mainly on finely grained sandy soils (Hartmann, 2001). The leaves are three-angled, spindle-shaped, quill-shaped or club-shaped (Smith et al., 1998) and five to ten times as long as thick (Hartmann, 2001). In their natural area of distribution, the plant habit of some *Cephalophyllum* species was found to be highly influenced by the amounts of received rainfall (Hartmann, 1983). Flowers often form in clusters and open mainly over midday and in the afternoon. Within *Cephalophyllum*, a wide range of flower colors can be found (Fig. 2), even with different combinations in one flower (Smith et al., 1998). The flowers have five sepals and 15 to 20 feathery stylodia. Ovaries and capsules are segmented into eight to 20 locules (Herre, 1971).

Fig. 2 *Cephalophyllum* genotypes II and I

1.2.3 *Lampranthus*

Within the genus *Lampranthus*, 277 different species names are known (Smith et al., 1998), but Klak (2001) presumed that the true number of species will decrease to 60-80 after revision. Most species are endemic to the wetter winter rainfall areas near the Cape of Good Hope (Hartmann, 2001) and occur in a variety of habitats with different soil types. Klak (2001) points out that many species are threatened by agriculture and urbanization. The plants are described as creeping to erect shrubs with smooth stems (Smith et al., 1998). Free or slightly fused leaves are formed in pairs and reach lengths of up to 50 mm. Flowers exhibit a large variety of colors (Smith et al., 1998) and are formed solitary or in dichasia. The flowers have diameters of up to 60 mm (Hartmann, 2001; Fig. 3) and their daily opening lasts from mid-morning to late afternoon (Smith et al., 1998). Ovaries and capsules are segmented into five to seven locules (Van Jaarsveld and De Pienaar, 2004). Generally, *Lampranthus* species tolerate drought, but are sensitive to frost (Smith et al., 1998).

Fig. 3 *Lampranthus* genotypes II and III

1.2.4 *Delosperma*

The genus *Delosperma* comprises approximately 100 species (Van Jaarsveld and De Pienaar, 2004), which, unlike the majority of other Aizoaceae genera, mostly occur in African summer rainfall regions with more than 400 mm annual precipitation (Hartmann, 2001). The plants are described as ground-covering to erect-shrubby (Van Jaarsveld and De Pienaar, 2004) with soft and fleshy, flat to terete leaves. Flowers mostly appear in dichasia and are separated by relatively long internodes. Different species are mainly distinguished by growth forms and leaf shapes, as the flower morphology within *Delosperma* is less diverse (Hartmann, 2001). Generally, the flowers have five stylodia and the ovaries are segmented in just as many locules (Herre, 1971). Many *Delosperma* species are long-living and some are known to tolerate frost (Van Jaarsveld and De Pienaar, 2004).

Fig. 4 *Delosperma* genotype I

1.3 Background and objectives of the research project

1.3.1 Determination of flower-inducing factors as a prerequisite for breeding approaches and horticultural production

A few highly succulent Aizoaceae taxa are mainly cultivated because of their extraordinary and bizarre morphology (e.g. *Lithops sp.* and *Conophytum sp.*). However, most other genera are of ornamental value because of their intensively colored flowers. For breeding approaches as well as for the development of cultivation strategies, knowledge of flower-inducing factors and their control in a greenhouse environment are required. Regarding flower induction in the Aizoaceae, only few information were available (e.g. Struck, 1994; Adams et al., 1998) and up to now, experiments have not been conducted in a horticultural context. Generally, five different but interacting pathways are known to control flowering in plants. These comprise impacts of temperature (vernalization and ambient temperature), photoperiod, gibberellin, endogenous regulators (autonomous pathway) and plant age (Srikanth and Schmid, 2011; Fig. 5). The natural habitats of all genera investigated in this thesis are influenced by seasonally varying climatic and light conditions (e.g. Le Roux et al., 1989; Hartmann, 2001). For this reason, photoperiod and temperature were selected as potential flower-inducing parameters in the performed experiments (Manuscript I).

Day length was the first flower-inducing factor that has been investigated in controlled experiments (Kobayashi and Weigel, 2007). Since the early studies on photoperiodic responses, which have been conducted almost 100 years ago (e.g. Garner and Allard, 1920), plants are commonly classified as (1) long day plants (LDP) in which flowering is induced when day lengths exceed a certain threshold and (2) short day plants in which flowering is induced when night lengths exceed a certain threshold. In a third group, referred to as (3) day neutral plants, photoperiods have no impact on flowering (Srikanth and Schmid, 2011). For a long time, however, the mechanisms by which plants measure day lengths and subsequently form flowers were only poorly understood. Today, the photoperiodic pathway is almost completely decrypted in some model plants with sequenced genomes such as *Arabidopsis thaliana* (LDP) or *Oryza sativa* (SDP). Plants possess an endogenous molecular timekeeper, also referred to as "circadian clock", which is regulated by environmental cues such as the day-night cycle (Johansson and Staiger, 2015). Generally, light signals of different wavelengths are perceived in leaves by the specialized photoreceptors phototropins, cryptochromes and phytochromes (e.g. Lariguet and Dunand, 2005). The endogenous timekeeper follows rhythms of approximately 24 hours, which were found to be divided in light-sensitive (photophile) and dark-sensitive (scotophile) phases (first described by Bünning, 1936). The measurement of day lengths by plants is based on whether light signals coincide with the photophile or scotophile phase. Dependent on the presence or absence of ambient light, circadian clock genes control the activity of the transcription factors CONSTANS (CO; in *Arabidopsis*) or its homolog HEADING DATE 1 (Hd1; in rice), which are the key components of the photoperiodic pathway (Johansson and Staiger, 2015). In *Arabidopsis*, CO activates the transcription of *FLOWERING LOCUS T* (*FT*; also known as "florigen") in phloem companion cells. Subsequently, the FT protein moves from the leaf to the shoot meristem, where it activates downstream regulators of flower formation such as APETALA1 (AP1). The homolog of *FT* in the short day plant rice was termed *HEADING DATE 3*. Although photoperiodic control of flowering is differently expressed by plants, the basic principles and the CO-FT module appear to be highly conserved (e.g. reviewed by Jung and Müller, 2009; Srikanth and Schmid, 2011; Johansson and Staiger, 2015). In horticultural production of ornamental plants, night interruptions by low-intensity lighting can accelerate flowering of LDPs and inhibit flowering of SDPs during seasons with short photoperiods (Craig and Runkle, 2016).

In many plant species, the onset of flowering requires a preceding prolonged exposure to low temperatures. This process, also known as vernalization, is best understood in *Arabidopsis* where it is regulated in parallel to the abovementioned photoperiodic pathway. Under non-inductive conditions, the MADS-box protein FLOWERING LOCUS C (FLC) represses the transcription of floral pathway integrators such as *LEAFY* (*LFY*), which encode proteins that activate floral meristem identity genes (e.g. *AP1*). In response to prolonged cold, *FLC* expression is downregulated by the proteins VERNALIZATION INSENSITIVE3 (VIN3), VERNALIZATION1 (VRN1), VRN2 and others, resulting in abolishment of floral repression (e.g. reviewed by Henderson and Dean, 2004). Balasubramanian et al. (2006) described a further temperature-controlled pathway in *Arabidopsis*, in which flowering is induced by elevated ambient temperatures. Although some genes that are involved in this pathway were identified (Balasubramanian et al., 2006), only little is known about how temperature is perceived by plants and thermosensor molecules have not been identified yet (Samach and Wigge, 2005).

For a long time, gibberellic acid (GA) was referred to as the flowering hormone, because Lang (1952) observed immediate onset of flowering in *Samolus parviflorus* and *Crepis tectorum* after application of GA. However, in experiments conducted thereupon, exogenous application of GA had no effect on flowering in other plants (Langridge, 1957). Today, the role of GAs in flower induction is best understood in *Arabidopsis*. In this plant, GAs regulate the expression of *FT* and other floral integrator genes or transcription factors, such as *LEAFY* (*LFY*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *AGAMOUS LIKE 24* (*AGL24*) (reviewed by Srikanth and Schmid, 2011).

The last flowering time pathway decrypted in *Arabidopsis* was the "aging pathway", which prevents plants from precocious flowering, but also ensures flowering under non-inductive conditions. In seedlings, the microRNA miR156 is highly abundant, but decreases in the course of plant development. This microRNA regulates the expression of SQUAMOSA PROMOTOR BINDING LIKEs (SPLs) transcription factors through transcript cleavage. During the adult developmental phase (when miR156 levels are low), SPLs activate miR172. This microRNA targets transcripts of genes, whose products are known to repress flowering, such as *APETALA 2* (*AP2*), *TARGET OF EAT 1* and *2* (*TOE 1* and *2*), *SCHLAFMÜTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*) (reviewed by Wang, 2014).

In several plant species native to South African ecosystems, including some Ruschioideae taxa, temperature was found to have an impact on the induction and development of flowers (e.g. Van Rooyen et al. 1991; Struck 1994; Dreyer et al. 2006; Ehrich et al. 2009, 2010; Thompson et al. 2011). The findings of these studies will be discussed in Chapter 2.

The investigations described in this thesis aimed at gathering first insights into the formation of flowers in *Cephalophyllum*, *Delosperma* and *Lampranthus* in order to evaluate the feasibility of their horticultural production in Central Europe. The obtained findings can contribute to the development of cultivation schedules.

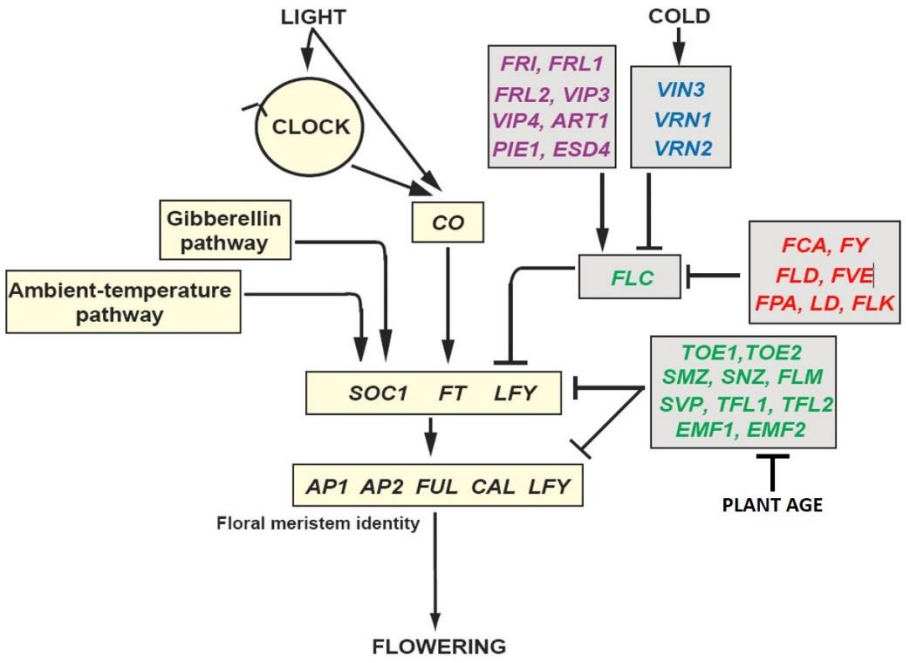

Fig. 5 Pathways controlling flowering time in *Arabidopsis*. Floral pathway integrators and meristem identity genes (black): *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), *APETALA1* and *2* (*AP1/2*), *FRUITFULL* (*FUL*), *CAULIFLOWER* (*CAL*), *CONSTANS* (*CO*). Floral repressors (green): *FLOWERING LOCUS C* and *M* (*FLC*, *FLM*), *TERMINAL FLOWER1* and *2* (*TFL1/2*), *SHORT VEGETATIVE PHASE* (*SVP*), *TARGET OF EAT1* and *2* (*TOE1/2*), *SCHNARCHZAPFEN* (*SNZ*), *SCHLAFMUTZE* (*SMZ*), *EMBRYONIC FLOWER1* and *2* (*EMF1/2*). Genes encoding proteins that promote *FLC* expression (purple): *FRIGIDA* (*FRI*), *FRIGIDA LIKE1* and *2* (*FRL1/2*), *PHOTOPERIOD INSENSITIVE EARLY FLOWERING1* (*PIE1*), *AERIAL ROSETTE1* (*ART1*), *EARLY UNDER SHORT DAYS4* (*ESD4*), *VERNALIZATION INDEPENDENCE3* and *4* (*VIP3/4*). Genes encoding proteins that downregulate the expression of *FLC* in response to prolonged cold (blue): *VERNALIZATION INSENSITIVE3* (*VIN3*), *VERNALIZATION1* and *2* (*VRN1/2*). Genes of the autonomous pathway (red): *FCA*, *FY*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* and *K* (*FLD*, *FLK*), *FVE*, *FPA* (Henderson and Dean, 2004, modified)

1.3.2 Localization and overcoming of hybridization barriers

Because the evolution of new taxa often originates in interspecific hybridization (Abbott, 1992), such events presumably contributed also to the hyperdiversity of the Aizoaceae. On the other hand, speciation generally involves the formation of reproductive isolation mechnisms. Depending on their mode of action, two different categories of hybridization barriers are distinguished: (1) Prezygotic barriers include spatial or temporal separation (e.g. in respect of flowering times), pollinator-specificity and specificity in pollen-pistil interactions. (2) Postzygotic barriers prevent the formation of seeds after fertilization or reduce the viability and/or fertility of the hybrid progeny (Rieseberg and Carney, 1998; Swanson et al., 2004).

Breeding programs of ornamental plants usually begin with the attempt to create new genetic variability and combinations of desirable traits through cross-pollinations. Unfortunately, the cross-compatibility of midday flowers is known to be limited and various manifestations of hybridization barriers were observed after combinations of distantly related taxa (Hammer and Liede 1990, 1991; Hammer 1995). Previous hybridization experiments of the cooperating breeding company only rarely resulted in seed formation or viable progeny. For this reason, the experiments described in this thesis primarily aimed at characterizing and overcoming the hybridization barriers that occur within and between the investigated genera. Determining the type of hybridization barrier was required to choose suitable methods to overcome them.

In practical plant breeding, several methods can be applied to detect and overcome prezygotic barriers. Divergent flowering times can be synchronized by adjusting climatic factors and photoperiods in the greenhouse. As this practice is difficult or even impossible in many plants (e.g. trees), storage of pollen under appropriate conditions until the seed parent starts flowering is often more convenient. However, the viability of pollen should be tested after harvest, to make sure that the pollen donor does not exhibit partial or complete male sterility, and after storage to ascertain, if pollen viability has declined. Commonly used laboratory methods to determine the viability of pollen grains are stainings with fluorescein diacetate (FDA) (established by Heslop-Harrison and Heslop-Harrison, 1970) or tetrazolium salts (e.g. Norton, 1966), which mainly focus on the enzyme activity in the vegetative cell and, in case of FDA tests, on cell membrane integrity (Heslop-Harrison and Heslop-Harrison, 1970). Furthermore, *in vitro* germination tests are widely acknowledged (e.g. Brewbaker and Kwack, 1963; Stanley and Linskens, 1985; Shivanna and Rangaswamy, 1992). These techniques have previously been tested with pollen of several midday flower genotypes and pollen germination tests turned out to give the most reliable results (Braun, 2012).

After pollination, various molecular interactions of the male gametophyte and the maternal tissues regulate the guidance of pollen tubes through the stigma towards the ovules (e.g. Dresselhaus and Franklin-Tong, 2013). Thus, when the molecular crosstalk fails, prezygotic hybridization barriers can occur at each point in time between pollen germination and sperm delivery. *In situ* pollen germination and tube growth can be observed microscopically by staining flowers with the fluorescent dye aniline blue at different times after pollination (established by Martin, 1959). Previous analyses of pollen tube growth *in situ* after intra- and intergeneric cross-pollinations of midday flower genotypes indicated that prezygotic barriers do not occur before ovule penetration (Braun, 2012). In other studies, the inhibition of pollen germination or tube growth has been successfully abolished by various methods such as pollination of flower buds before anthesis, pollination of styles after removal of the stigma (Bhat and Sarla, 2004), grafting of pollinated stigmas on styles of the incongruent seed parent (Van Tuyl et al., 2000), application of mentor pollen (Singsit and Hanneman, 1991) or *in vitro* pollination (Zenkteler, 1990). However, prezygotic barriers can also occur inside the ovule during tube burst and sperm delivery. These processes, which are also referred to as pollen tube reception, as well depend on molecular interactions (Kessler and Grossniklaus, 2011). Because the observation of pollen tube growth using the aniline blue staing method only allowed the detection of prezygotic barriers before ovule penetration (Braun, 2012), ovule clearing and differential interference contrast (DIC) microscopy were used to analyse the subsequent events in the experiments described below.

After fertilization, defective development or degeneration of the endosperm and undersupply with nutrients frequently lead to embryo abortion (Raghavan, 2006). As defective endosperm development is often caused by diverging genome sizes or chromosome numbers of the parents (Haig and Westoby, 1991), polyploidizations could restore the normal genomic ratio of 2m:1p (e.g. Bushell et al., 2003). Furthermore, Bateson-Dobzhansky-Muller (BDM) incompatibilities, which rely on deleterious

interactions of diverged parental transcripts (Rieseberg and Carney, 1998; Lafon-Placette and Köhler, 2015) as well as epigenetic changes (Lohe and Chaudhury, 2002; Michalak, 2009) are regarded as reasons for hybrid dysfunctions and endosperm failures. Late-acting postzygotic barriers still can occur after seed development and even in the F_1 or later generations. These barriers become manifest in low hybrid vigor, hybrid breakdown, albinism or hybrid sterility (Bomblies and Weigel, 2007; Greiner and Bock, 2013). Of these, at least hybrid sterility can be circumvented sometimes through polyploidizations (Van Tuyl and Lim, 2003; see also Chapter 1.3.3)

Because previous microscopic observations after intra- and intergeneric cross-pollinations within *Delosperma* and *Lampranthus* have shown that the pollen tube growth towards the ovules was not inhibited (Braun, 2012), the investigations of this thesis focused on analyses of fertilization and early embryogenesis. Aside from the characterization of hybridization barriers, differential interference contrast (DIC) microscopy was also used to clarify the question of self-compatibility. Principally, one possibility to overcome postzygotic hybridization barriers is the application of the embryo rescue method (Winkelmann et al., 2010), which, apart from conventional cross-pollinations, was also performed in the investigated genotypes. Because progeny resulting from hybridization experiments could as well originate from self-fertilization or apomixis, the hybrid state should be verified, e.g. by molecular markers (Winkelmann et al., 2010). For this purpose, the amplified fragment length polymorphism (AFLP; established by Vos et al., 1995) technique was utilized in the present thesis. The data obtained with this method were furthermore used to calculate genetic differences between the parental genotypes as well as the degrees of similarity between the parents and their progeny.

1.3.3 The mechanisms of polyploid formation in plants

Polyploid organisms possess three or more complete sets of chromosomes within their cells (Ramsey and Schemske, 1998). The occurrence of polyploidy is widespread among plants and recent phylogenomic analyses revealed that whole-genome duplications (WGD) have already taken place in ancestral lineages of both angiosperms and gymnosperms (Jiao et al., 2011). Generally polyploidy is regarded as an important evolutionary mechanism because it enables the development of novel physiological and morphological characteristics and contributes to the reproductive isolation of plant species (Ramsey and Schemske, 1998; Schatlowski and Köhler, 2012). Among others, advantageous effects of polyploidization events on plants are mutation buffering, dosage effects, increased allelic diversity and heterozygosity, and neofunctionalization of duplicated genes (Comai, 2005; Te Beest et al., 2012). However, the polyploidization process is generally accompanied by extensive genetic and epigenetic changes that result in severe genome reorganization and may have negative effects on fertility and viability (Chen, 2010).

Regarding the formation of naturally occurring polyploids, two completely different ways are distinguished: somatic chromosome doubling (somatic polyploidization) and gametic nonreduction (sexual polyploidization) (De Storme and Geelen, 2013a). Spontaneous somatic chromosome doubling through endomitosis or nuclear fusion occurs, for instance, in meristem tissues, in wounds, in zygotes or in young embryos, but only little is known about the frequency of such events (Ramsey and Schemske, 1998; De Storme and Geelen, 2013a). In plant breeding, somatic polyploidizations are often induced by antimitotic agents, such as colchicine, oryzalin or trifluralin (e.g. Dhooghe, 2009). Polyploidization approaches are mainly conducted to generate new morphological and genetic variability (e.g. Tavan et al., 2015), to overcome ploidy-related hybridization barriers (Schatlowski and Köhler, 2012) and self-incompatibility (e.g. Cohen and Tel-Zur, 2012) or for restoration of fertility (Urwin, 2014).

In an evolutionary context, meiotic non-reduction and union of unreduced gametes are believed to occur much more frequently than somatic chromosome doubling, and therefore can be regarded as the driving forces in polyploid formation (Ramsey and Schemske, 1998). In addition, 2n gametes have also been discovered as valuable tools in breeding programs (Younis et al. 2014), because their utilization can result in progeny with increased heterozygosity and allelic diversity (Bretagnolle and Thompson, 1995). Generally, three different developmental-specific classes of 2n gamete formation are distinguished: pre- and post-meiotic genome doubling and meiotic restitution. Pre-meiotic genome doubling (e.g. in pollen mother cells) is assumed to occur rarely in plants, as it has been only observed in a few instances (e.g. Lelley et al., 1987; Falistocco et al., 1995; De Storme and Geelen; 2013b). The same seems to be true for post-meiotic doubling, a mechanism in which homozygous 2n gametes are formed by spontaneous genome duplication in haploid spores. In the course of meiotic restitution, which is widely acknowledged as the major route of 2n gamete formation, meiotic cell division is converted into a mitosis-like nonreductional process. Unreduced gametes formed by an omission of meiosis I are genotypically identical to the parent because recombination is abolished and chromosomes undergo directly an equatorial division. Thus, 2n gametes generated by first division restitution (FDR) usually retain paternal heterozygosity. In contrast, 2n gamete formation through second division restitution (SDR) allows chromosome pairing and recombination during meiosis I, leading to reduced levels of heterozygosity in the meiotic products (De Storme and Geelen, 2013a; Fig. 6). According to Ramanna and Jacobsen (2003), the formation of unreduced gametes is primarily controlled genetically, but the rates of their occurrence are also influenced by environmental factors.

In breeding approaches, 2n gamete formation was successfully stimulated by high (e.g. Negri and Lemmi, 1998; Pécrix et al., 2011) and low temperatures (e.g. Mason et al. 2011) as well as by the application of chemicals such as colchicine, oryzalin, trifluralin or N_2O (Younis et al. 2014). Due to their higher number and easier accessibility, most investigations on meiotic nonreduction focus on male gametes. The most commonly applied methods to test for the presence of unreduced pollen grains are morphological screenings (as 2n pollen grains were often found to be larger than reduced ones), progeny analysis of interploidy crosses and analysis of microsporogenesis (Bretagnolle and Thompson, 1995). In a few instances also flow cytometry was used to screen for unreduced male gametes (Van Tuyl et al., 1989; Bino et al., 1990; Kron and Husband, 2015).

Suehs et al. (2006) detected macro pollen in different species of the Ruschioideae genus *Carpobrotus*. Although no polyploid specimens were found in this genus so far, meiotic abnormalities (microspore diad and triad formations) observed by Diadema (2002) suggest that *Carpobrotus* macro pollen were diplogametes (Suehs et al. 2006). Varying grain diameters, indicating spontaneous formation of unreduced male gametes in the genotypes investigated in this thesis, were already observed during first pollen analyses. For this reason, various methods were applied to verify the presence of 2n gametes and to determine the rates of their formation (Chapters 4 and 5). Another objective was to identify the mechanisms of 2n gamete formation with the intention to develop strategies that promote their occurrence. The obtained findings primarily illustrate the suitability of commonly applied screening methods in the investigated Aizoaceae genera.

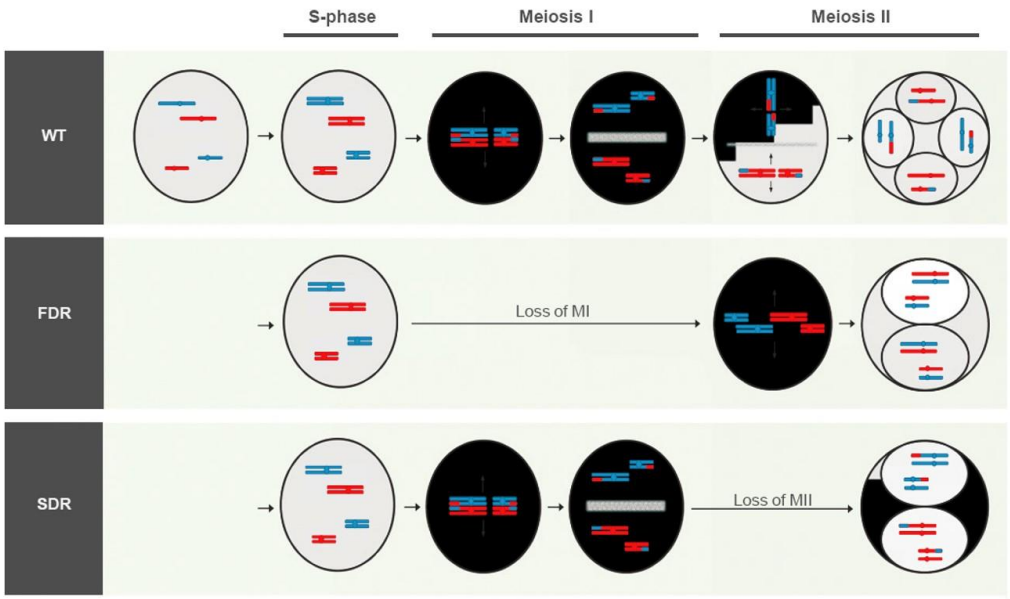

Fig. 6 Formation of 2n gametes by meiotic nonreduction; red and blue bars depict chromosomes obtained from genetically different parents; FDR, first division restitution; MI, II, meiosis I and II; SDR, second division restitution; WT, wild type (De Storme and Geelen, 2013a, modified)

1.3.4 Estimation of genome sizes and characterization of endoreduplication patterns

Aside from the abovementioned mechanisms of polyploidy, another cytological process referred to as endoreduplication (or endoreplication, endocycling) also generates higher DNA contents of cells. In this mode of cell cycle, nuclear DNA amounts are doubled repeatedly in the absence of cytokinesis leading to polyteny (D´Amato, 1984; Breuer et al., 2014). This means in contrast to endomitosis, that the original chromosome number is retained during endocycling because the sister chromatids do not segregate after DNA replication (Šesek et al., 2005; Bauer and Birchler, 2006; Bourdon et al., 2011; Fig. 7). During endoreduplication, molecular regulators, which also hold key positions in mitosis, such as cyclins (CYC), cyclin-dependent kinases (CDK) and CDK inhibitors are modified transcriptionally and/or post-translationally (Breuer et al., 2014). Endoreduplication is assumed to occur in the majority of angiosperm species, but often the process is restricted only to certain tissues or cell types (polysomaty) (D´Amato, 1984). According to Barow and Meister (2003), endoreduplication was frequently detected in annual and biennial species, less often in perennials and it seems to be absent in woody plants. Furthermore, the extent of endoreduplication primarily depends on the taxonomic position of a plant (Barow and Meister, 2003). The physiological functions of endoreduplication are presumably diverse. As nuclear DNA contents and cell volumes are positively correlated in angiosperms (Jovtchev et al., 2006), endoreduplication accelerates growth of plant organs through increased cell expansion (e.g. Hayashi et al., 2013) and facilitates the development of water-storing tissues in drought-adapted plants (De Rocher et al., 1990; Del Angel et al., 2006). Furthermore, endoreduplication can compensate for a lack of nuclear DNA in a wide range of cells or tissues with high metabolic demands (e.g. embryo suspensor or endosperm cells) (D´Amato, 1984).

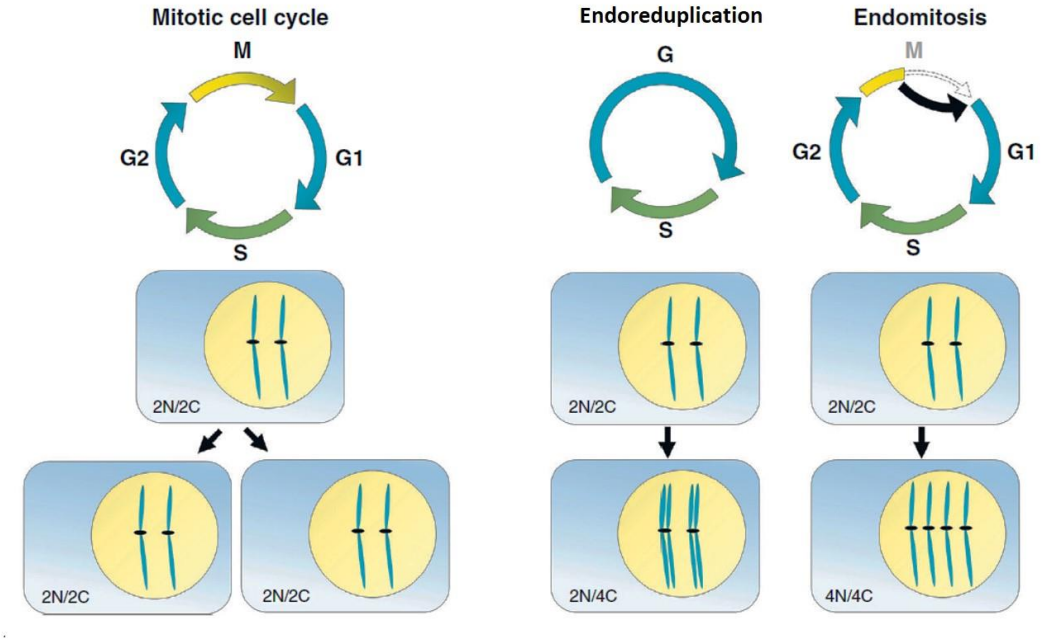

Fig. 7 Deviations from the mitotic cell cycle; letters denote chromosome sets (N), C-values (C), and cell cycle phases (M, S, G1 and G2) (Breuer et al., 2014, modified)

Only little is known about the performance of polysomatic tissues in *in vitro* polyploidization approaches, which include shoot regeneration from single cells. For a long time, cells containing polytene chromosomes were regarded as unable to divide mitotically (D´Amato, 1964; Nagl, 1981), suggesting that they are unsuitable for plant regeneration *in vitro*. However, Valente et al. (1998) demonstrated that simultaneous application of auxins and cytokinins could provoke multiple amitotic divisions of endoreduplicated tobacco cells, which ultimately re-entered mitosis. However, at least from studies in insects and mammals is known that amitotic cell divisons often lead to severe genomic abberations, which contribute to diseases such as cancer (Fox et al., 2010; Fox and Duronio, 2013). De Rocher et al. (1990) characterized the annual midday flower *Mesembryanthemum crystallinum* flow cytometrically and revealed organ-specific endoreduplication patterns. Meiners et al. (1991) used various parts of this species for *in vitro* organogenesis and regenerated fertile plants with unaltered DNA content. However, the obtained regeneration frequencies were rather low, particularly in leaf explants. Higher regeneration rates in *M. crystallinum* were obtained through somatic embryogenesis by Cushman et al. (2000). In both studies, however, endoreduplication has not been discussed as crucial factor for the success of *in vitro* organogenesis approaches. The characterization of endoreduplication described in this thesis mainly aimed at providing selection criteria for explants used in future tissue culture trials. Presumably, *in vitro* organogenesis is more efficient in explants that predominantly consist of cells with 2C DNA content. The application of antimitotic agents may give rise to shoots with a basic DNA content of 4C.

Up to now, endoreduplication has not been considered as a selection criterion in ornamental plant breeding. However, the very same was suggested by Agulló-Antón et al. (2013) after revealing a positive correlation between the occurrence of endoreduplication and petal size in carnation cultivars.

Previous flow cytometric analyses in some genotypes of the investigated genera revealed, that their leaf tissues consisted of cells with up to five redoubling nuclei sizes. At this time, the diverging DNA contents of cells hampered estimations of genome sizes, because peaks (display of detected fluorescence intensities) formed by endoreduplicative nuclei frequently overlapped the peak of internal standards (Braun, 2012). Therefore, optimization of the methodology used for genome size estimations was an additional objective of the research project. As gametophytic cells were supposed to be non-endoreduplicative, flow cytometric measurements of pollen nuclei were tested as an alternative strategy. So far, genome size data has been only available for the Mesembryanthemoideae species *M. crystallinum*, which possesses a very low nuclear DNA content of approximately 0.9 pg/2C (De Rocher et al., 1990; Bennett and Leitch, 1995).

1.3.5 General background and objectives

The studies in this thesis were conducted as part of the cooperation project "Development of horticultural and biotechnological methods for breeding purposes within the Aizoaceae", which was partly financed by the German Federal Ministry for Economic Affairs and Energy within the framework of the program "Zentrales Innovationsprogramm Mittelstand (ZIM)" [grant number KF2508005MD2]. The research project was initiated in cooperation with the company Klemm & Sohn GmbH & Co. KG (Stuttgart, Germany, part of the Secta One group), a breeder and producer of ornamental plants. The cooperation partner intended the development of novel Aizoaceae cultivars to broaden its range of products in the market segment of bedding plants. Midday flowers (common name for members of the Aizoaceae family) have potential for a wider use as ornamental plants, particularly because of their intensively colored flowers, a remarkable diversity of growth forms and relatively high tolerance to drought. However, due to various forms of cross-incompatibilities and long juvenile phases (Hammer and Liede 1990, 1991; Hammer 1995), breeding within the Aizoaceae is not a simple task. Furthermore, cultivation schedules that enable efficient greenhouse production are underreported up to now. Any breeding project requires intimate knowledge of different reproduction-related aspects in the plants of interest. However, issues such as flower induction or the underlying principles of hybridization barriers were hardly investigated in the plant family Aizoaceae. First insights in the flower biology and seed development of some midday flower genera with attractive traits for plant breeding were already gathered in the context of a master thesis (Braun, 2012). In particular, observations of unimpaired pollen tube growth after wide cross-pollinations opened new perspectives and offered incentives to continue the investigations. The agenda of the abovementioned research project comprised the following objectives:

- Determination of flower-inducing factors and of how these could be controlled in a greenhouse environment
- Localization and overcoming of hybridization barriers after intra- and intergeneric cross-pollinations
- Characterization of self-incompatibility mechanisms
- Identification of putative spontaneously formed unreduced male gametes and an evaluation of how these could be utilized for polyploidizations or overcoming of ploidy-related hybridization barriers
- Estimation of genome sizes by flow cytometric analyses
- Characterization of endoreduplication (endopolyploidy) as a prerequisite for *in vitro* polyploidizations

Altogether, these work packages aimed at providing the basis for midday flower breeding programs.

Chapter 2: Manuscript I

Impacts of photoperiod and temperature on flower induction in *Delosperma, Lampranthus* and *Cephalophyllum* (Aizoaceae)

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Original article

Impacts of photoperiod and temperature on flower induction in *Delosperma*, *Lampranthus* and *Cephalophyllum* (Aizoaceae)

Philipp Braun and Traud Winkelmann

Abstract

Midday flowers (Aizoaceae), whose origin and main area of distribution are located in southern Africa, represent the most species-rich family of succulent plants. Because of their intensively colored flowers, a high diversity of growth forms and relatively high tolerance to drought, several Aizoaceae species are attractive candidates for breeding approaches or direct use as ornamental plants. Despite their advantageous characteristics, midday flowers are rarely offered for sale in Central Europe and only a few commercially traded cultivars were developed so far. A wider use of midday flowers as ornamental plants requires intimate knowledge of means to control their cultivation (e.g. control of flowering time), which is underreported up to now. The present study aimed at determining the effects of different photoperiods and temperatures on flower induction in the genera *Delosperma*, *Cephalophyllum* and *Lampranthus* in two separate experiments. Day length could be ruled out as a flower-inducing factor, because flower organs were observed irrespective of photoperiods in genotypes of all three genera. However, only plants cultivated under long-day conditions (16 hours of daily exposure to light) had a desirable appearance, which was reflected in significantly higher dry matter contents. Cultivation in climate chambers for several weeks revealed distinct impacts of mean daily temperatures (MDT) on flower formation. The investigated *Delosperma* genotype tended to develop more flowers at 20 °C than in two variants in which the plants were exposed to 14 °C MDT. In contrast, cultivation at 14 °C MDT resulted in significantly higher flower numbers in two *Lampranthus* genotypes. High numbers of flowers were found in the investigated *Cephalophyllum* genotype at both MDTs and significant differences were not detected.

Keywords

flowering time control, mean daily temperature, midday flowers, novel ornamentals, photoperiodism, succulent plants

Significance of the study

What is already known on this subject?

Flower induction of Aizoaceae species has not been investigated in a horticultural context before. Flower induction is influenced by water availability and temperature in plants of arid South African ecosystems.

What are the new findings?

The investigated genotypes are neither obligate short-day plants nor long-day plants. In *Lampranthus*, flower induction was stimulated by low-, and in *Delosperma* by elevated mean daily temperatures.

What is the expected impact on horticulture?

The study contributes to the development of cultivation schedules and provides information about the feasibility of production of Aizoaceae in Central Europe.

Introduction

The Aizoaceae, which covers a total of more than 1800 species, is the largest of all succulent plant families. Its origin and main area of distribution is located in the South African Greater Cape Floristic Region (GCFR) (Klak et al., 2003), which consists of different biomes (Milton et al., 1997) with diverging climatic conditions (Peel et al., 2007). Midday flowers (common name for members of the family) occur in numerous growth and life forms and many drought tolerant species possess attractive, intensively colored flowers. Despite the prevalent popularity of some highly succulent genera (e.g. *Lithops*), which are occasionally offered as potted plants, the genetic resources of midday flowers were left largely unexploited by plant breeders and producers up to now. Unfortunately, this is also true for many other South African taxa with desirable features for floriculture (Reinten et al., 2011). One reason for the low-level presence of midday flowers on commercial markets (particularly in Central Europe) is that cultivation methods are underreported. Most producers of ornamentals wish to sell blooming plants at fixed dates. The achievement of this objective requires knowledge of flower inducing factors, which have not been determined in most Aizoaceae species so far. Our collection of Aizoaceae genotypes, which was maintained in a greenhouse at Leibniz Universität Hannover from 2011-2015 showed vigorous growth when cultivated under long-day conditions (photoperiods of at least 16 hours) at relatively stable mean daily temperatures of 20 °C. However, the majority of plants, which belong to the genera *Cephalophyllum*, *Lampranthu*s and *Delosperma*, formed relatively low numbers of flowers throughout the year and seasonal flowering periods were not identifiable. Obviously, cues which naturally trigger flowering in these succulents were missing in the artificial greenhouse environment. Due to the fact that to our knowledge experimental approaches on flowering of Aizoaceae species have so far not been conducted in a horticultural context, learning about the environmental conditions and the seasonal onset of bloom in the plants´ natural habitat is the only mean to limit the number of potential flower inducing factors. *Cephalophyllum* and *Lampranthus* occur in the southwestern part of South Africa (Hartmann, 2001), which is characterized by winter rainfall and, depending on the exact position, mediterranean, cold semi-arid or cold desert climate (Peel et al., 2007). The genus *Delosperma* is more widely distributed in summer rainfall regions (Hartmann 2001), which are mainly influenced by subtropical or oceanic climates (Peel et al., 2007). In Namaqualand (northwestern Cape), which is a diversity hotspot of the family Aizoaceae (Cowling et al., 1999), the flowering period of the vast majority of plant species lasts from August to October (Le Roux et al., 1989). During that time (and the preceding months) day lengths mainly fall below 12 hours, indicating a conceivable photoperiodic impact on flower induction.

The aim of the present study was to gather first insights of how photoperiod and temperature impact the formation of flowers in the three Aizoaceae genera. Follow-up experiments can contribute to the development of cultivation schedules for their use as bedding plants and provide information about the feasibility of production in Central Europe.

Material and Methods

Plant material and general culture conditions

Various genotypes of the Aizoaceae genera *Delosperma*, *Lampranthus* and *Cephalophyllum* (Fig. 1) were chosen for the two experimental set-ups described below. *Delosperma* genotype II, which shared many morphological characteristics with *D.* genotype I, is a traded cultivar (`Ruby´) developed by the Japanese breeder Koichiro Nishikawa. Both investigated *Cephalophyllum* genotypes largely corresponded to species descriptions of *C. alstonii* (syn. *franciscii*), and *Lampranthus* genotype II to

descriptions of the species *L. aureus* (e.g. Van Jaarsveld and De Pienaar 2004). The three genera form actinomorphic hermaphroditic flowers with inferior ovaries. Aside from various pigmentations and genus-specific morphological traits, the flowers differed most obviously in size. Flower diameters ranged from 10-18 mm in *Delosperma*, from 30-45 mm in *Cephalophyllum* and from 40-60 mm in *Lampranthus*.

Those plants, which served as donors of cuttings used in the experiments, were cultivated in peat/sand substrate (3:1) at 20 °C mean daily temperature. Relative humidity in the greenhouse was adjusted to 40-60 % by automatic ventilation. High pressure sodium lamps (Philips MASTER Agro 400 W) were used to ensure minimum daily photoperiods of 16 hours and for supplemental lighting when solar radiation fell below 25 klx. The plants were irrigated manually and fertilized once a week with 0.1 % Hakaphos rot© (8 % N: 12 % P_2O_5: 24 % K_2O, Compo, Münster, Germany). **[Figure 1]**

Evaluation of photoperiodic impacts

Cuttings of the genotypes *Delosperma* I, *Cephalophyllum* I, *Lampranthus* I and *L.* II were harvested from non-flowering donor plants in February 2013 and rooted in peat/sand substrate (3:1) at 20 °C mean daily temperature (MDT), high relative humidity and photoperiods of 12 hours. After 15 days, the rooted cuttings were potted in 11 cm-pots and distributed to three neighboring greenhouse chambers in which different photoperiodic regimes were realized (referred to as regime P1, P2 and P3 below):

Regime P1: 16 hours light/ 8 hours darkness

Regime P2: 9 hours light/ 15 hours darkness

Regime P3: 9 hours light/ 15 hours darkness + 2 hours night interruption

In all greenhouse chambers, mean daily temperatures (MDT) were adjusted to 20 °C by automatic ventilation, and high pressure sodium lamps were used to extend natural photoperiods (regime P1) and for supplementary lighting when solar radiation fell below 25 klx. In regime P3, night interruption was given by two fluorescent lamps (36 W, Osram) for two hours in the middle of the dark period. Irrigation and fertilization were applied as described above.

For each of the photoperiodic regimes, twelve plants per genotype were randomly chosen and divided in three groups (replicates) of four plants. These groups were randomly positioned on greenhouse tables and, in order to minimize possible edge effects, the experimental set-ups were surrounded by additional plants of a further *Lampranthus* genotype. During nine weeks, numbers of open flowers formed by each plant (and numbers of flower buds in *Cephalophyllum* and *Lampranthus*) were documented weekly. At the end of the experiment, fresh masses and, after drying at 80 °C for seven days, dry matters of aboveground plant parts were determined.

Evaluation of temperature impacts

In May 2014, cuttings of the genotypes *Delosperma* II, *Cephalophyllum* II, *Lampranthus* I and *L.* II were rooted as described above and potted in 8 cm-pots. Twelve plants of each genotype (divided in three groups of four plants) were set up in three different climate chambers, respectively. Again, possible edge effects were avoided by margins of additional plants. Each climate chamber was equipped with four high pressure sodium lamps (Philips MASTER Agro 400 W), which were switched on for a daily duration of 16 hours. Light intensities (PPFD) at plant level ranged from 100 to 130 µmol m^{-2} s^{-1}. Temperature regimes in the different chambers were as follows:

Regime T1: 14 °C steady

Regime T2: 14 °C MDT; 18 °C day/ 6 °C night

Regime T3: 20 °C steady

During 13 weeks, numbers of flower buds, open flowers and wilting flowers formed by each plant were documented weekly. Wilting *Cephalophyllum* and *Lampranthus* flowers were labeled after acquisition to avoid wounding. Because *Delosperma* flowers drop off soon after wilting, they were removed after acquisition. Afterwards, all plants were collectively transferred to one greenhouse (20 °C MDT; culture conditions see above), where the evaluation was continued for another two weeks. Because flowering was further monitored also during the subsequent weeks, aboveground fresh mass and dry matter content were not determined.

Statistical analysis

Data obtained for aboveground fresh mass and dry matter content in experiment 1 as well as numbers of flower buds formed in experiment 2 were checked for homogeneity of variance and compared using Tukey tests (R-software version 3.0.2) to determine statistically significant differences ($\alpha = 0.05$). Numbers of flowers recorded in experiment 1 were not analyzed statistically, because firstly many plants of the two *Lampranthus* genotypes did not form any flowers (zero values) and secondly the experiment was carried out to identify obligate photoperiodic effects (yes or no reaction).

Results

Effect of the photoperiod

First flower buds were recorded in *Delosperma* I and *Cephalophyllum* I under all photoperiodic regimes after one week. Until the third week of the experiment, morphological differences between the *Delosperma* plants were not obvious and the formation of flower buds appeared to occur evenly in the three variants. From the fourth week onwards, *Delosperma* plants, which were cultivated under long-day conditions (regime P1), exhibited a more compact habit, branches with higher stability and smaller leaves than those cultivated under regimes P2 and P3. Flower buds were continuously formed in all variants till the end of the experiment and the highest average number of open flowers per plant was documented under regime 1 (Table 1). Also in *Cephalophyllum* I flowers were continuously formed irrespective of the photoperiodic regime. The average number of documented flowers per plant was much lower than in *Delosperma* and highest under regime P2 (4.0) and lowest under regime P3 (2.3; Table 1). *Cephalophyllum* plants which were cultivated under short-day conditions (regimes P2 and P3) had softer leaves and slightly reduced flower diameters in comparison to those cultivated under long-day conditions (regime P1). First flower buds of *Lampranthus* I were observed under regimes P2 and P3 after one week. In these variants, formation of further flower buds was not recorded until the fourth week of the experiment. Plants of *Lampranthus* I, which were cultivated under long-day conditions (regime P1), formed first flower buds after four weeks. The average total numbers of open flowers per plant were higher under regimes P1 and P3 (0.6) than under regime P2 (0.2; Table 1). In *Lampranthus* II, first flower buds were observed after one week under regime P1. The average number of documented open flowers per plant in this variant was 0.5 at the end of the experiment (Table 1). After three weeks, *Lampranthus* II plants formed a few flower buds also under short-day conditions (two buds under regime P2 and one bud under regime P3). These buds were much smaller (approx. 5-

10 mm in length) than those formed under regime P1 (approx. 20-25 mm) and dried off before anthesis. **[Table 1]**

The determined fresh masses of aboveground plant parts are displayed in Figure 2. In *Delosperma* I and *Cephalophyllum* I, the fresh masses of plants which were cultivated under regime P2 were significantly higher than those of the other variants. Also in both *Lampranthus* genotypes cultivation under regime P2 resulted in the highest fresh masses, but the differences to the other treatments were not statistically relevant. More distinct differences between the three photoperiodic regimes were found after determination of dry matter contents. In all investigated genotypes, plants cultivated under long-day conditions (regime P1) showed significantly higher dry matter contents (approx. 10-12 % on average) than the plants which were grown under regimes P2 and P3 (approx. 5-9 % on average; Fig. 2). All plants, which had received night interruptions (regime P3) had higher dry matter contents than those, in which dark periods were not interrupted (regime P2), but statistically significant differences between these treatments were only found in *Delosperma* I, *Cephalophyllum* I and *Lampranthus* II (Fig. 2). **[Figure 2]**

Temperature effects

Some cuttings of *Delosperma* II had formed flower buds already during the rooting phase. For this reason, the average numbers of flower buds per plant ranged from 0.17 (20 °C MDT) to 0.25 (both 14 °C MDT variants) at the beginning of the experiment. Plants of this genotype formed flower buds in each variant during the duration of the experiment (Fig. 3). While the formation of flower buds occurred almost identical in both 14 °C MDT treatments, the bud set in *Delosperma* plants cultivated at 20 °C appeared to be faster from week six onwards. However, statistically significant differences between the regimes were not found before week eleven. From week 13 onwards, no newly formed flower buds were documented in regime T3. At the end of the experiment, average numbers of formed flower buds per plant ranged from 2.9 (regime T2) to 8.2 (regime T3). Although the average number of buds per plant formed under regime T1 (4.1) was lower than under regime T3, significant differences were only detected between regime T2 and regime T3 (Fig. 3). Plants grown at 14 °C MDT showed very compact habits, while those cultivated at 20 °C formed longer shoots and internodes (Fig. 4A).

In *Cephalophyllum* II, flower buds were evenly formed irrespective of the temperature regime. After 15 weeks the average number of flower buds formed per plant ranged from 7.7 (20 °C) to 8.6 (18 °C day-/6 °C night temperature; regime T2) and significant differences were not detected. At 20 °C, the leaves of *Cephalophyllum* plants were obviously longer than at cooler temperatures (Fig. 4B)

In *Lampranthus* I, first flower buds were observed after seven weeks at 20 °C and after nine weeks in both 14 °C MDT variants. After transfer to greenhouse conditions (week 13), the numbers of visible flower buds increased rapidly in those plants, which were previously cultivated at 14 °C MDT (regimes T1 and T2). At the end of the experiment, the average numbers of flower buds per plant in these variants were 8.5 (regime T1) and 8.9 (regime T2). Plants, which were grown at 20 °C, formed significantly lower numbers of flower buds (5.4 buds per plant on average; Fig. 3). Most plants cultivated at 20 °C had a less compact growth habit than at 14 °C MDT. In the cooler variants, all *Lampranthus* I plants exhibited partial leaf yellowing, which was initially observed at the leaf tips (Fig. 4C).

In *Lampranthus* II, first flower buds were observed after eight weeks under regime T1 and after nine weeks under regime T2. Only a single bud was observed at 20 °C during the whole experiment. Final

average numbers of formed flower buds per plant were 2.5 in both 14 °C MDT treatments. In contrast to the other investigated genotypes, differences in plant growth were not distinct in *Lampranthus* II (Fig. 4D). **[Figures 3 and 4]**

Discussion

Generally, five different but interacting pathways are known to control flowering in plants. These comprise impacts of temperature (vernalization and ambient temperature), photoperiod, gibberellin, endogenous regulators (autonomous pathway) and plant age (Srikanth and Schmid, 2011).

The investigated genotypes are neither obligate short-day plants nor long-day plants

As the flowering periods of most plants native to the northwestern Cape last from August to October (Le Roux et al., 1989) when day lengths mainly fall below 12 hours, a photoperiodic impact on flower induction in the investigated genera was conceivable. For the analyzed genotypes this assumption was disproved, as all of them formed flowers (or buds) (Table 1) irrespective of the photoperiod. Particularly because the plants received different daily light integrals during the experiment, the obtained data are insufficient to identify facultative photoperiodic impacts on flower induction. Nevertheless, a classification of the analyzed midday flowers as obligate long- or short-day-plants can be definitely ruled out. In all genotypes, plants cultivated under short-day conditions (regime P2) had the highest average fresh masses and statistically relevant differences to the other variants were found in *Delosperma* and *Cephalophyllum* (Fig. 2). Morphological differences were observed between the plants cultivated under long-day conditions (regime P1) and those which had received lower daily light integrals (regimes P2 and P3). Leaves and shoots of *Delosperma* and *Cephalophyllum*, which were formed under regimes P2 and P3, were much softer and less compact than under long-day conditions. Furthermore, under regimes P2 and P3, flowers of *Cephalophyllum* were smaller and dwarf flower buds of *Lampranthus* II discontinued their development before anthesis. The highest dry matter contents were found in plants cultivated under regime P1. Although this could be explained by higher received daily light integrals alone, significant differences of dry matter contents were also found between regimes P2 and P3. As the low-energy compact fluorescence lamps, which were used for night interruption in regime P3, only marginally contributed to the daily light integrals, the increase of dry matter contents in this variant can presumably be ascribed to a photoperiodic response. According to Adams and Langton (2005), dry masses of several plant species are higher under long-day conditions even when the plants receive identical daily light integrals, but the physiological reasons have not been detected yet. Under Central European conditions the production of the investigated midday flowers probably requires high daily light integrals, which can only be realized through assimilation lighting during winter and early spring.

Mean daily temperature influences flowering

For a duration of three years, Struck (1994) documented the flowering periods of 112 plants species in certain areas of Namaqualand with the aim to determine the relevant flower inducing factors in this ecosystem. The range of investigated plants comprised 14 Aizoaceae species, of which *Cheiridopsis denticulata*, *Leipoldtia schultzei* and four *Ruschia* taxa belong to the subfamily Ruschioideae. The flowering periods of these plants lasted from August-October (except for *Ruschia robusta*; Oct.-Nov.), but the exact dates for the onset of flowering varied each year. Struck (1994) found no correlation between the precipitation patterns and flowering times. In contrast, the time point of the first temperature drop in autumn (March/April) influenced the onset of bloom. Between March (start of the growing period after dry and hot summer months) and July/August (onset of flowering) mean

temperatures decreased from ~26 °C to ~12 °C (Struck, 1994). Also in the present study, lower temperatures had a positive impact on the formation of flower buds in two *Lampranthus* genotypes (Fig. 3). Because no differences with regard to the number of flower buds were found between regimes T1 and T2, mean daily temperatures rather than absolute temperature values appear to be crucial for flower induction. Although their final number was lower than in regimes T1 and T2, first flower buds of *Lampranthus* I were observed at 20 °C and also the duration between the occurrence of buds and anthesis was shorter under regime T3. Shortly after the transfer of plants from regime T1 and T2 to a greenhouse with 20 °C MDT, numerous flower buds, which must have been induced during the cultivation in climate chambers, became visible (Fig. 3). These observations can be explained by accelerated growth at higher temperatures and emphasize the importance to distinguish between flower induction and development in the context of flowering. In horticultural practice, synchronized appearance of *Lampranthus* flowers could be obtained by elevating the temperature level after well-defined periods, in which the plants were cultivated in cooler environments. In addition, microscopic analyses of axillary meristems could help to determine the exact time of flower induction.

In *Cephalophyllum*, flower bud numbers and the rates of their formation appeared to be unaffected by the chosen temperature regimes. For this reason, future experiments should include higher and lower mean daily temperature variants in order to define appropriate ranges for the cultivation of this genus. During studies in South African winter rainfall regions, Hartmann (1983) detected strong correlations between precipitation patterns and morphological features (e.g formation of runners) in some *Cephalophyllum* species. Whether the availability of water also influences flowering during the cultivation of this drought-adapted genus is unknown and should be investigated in coming trials.

Although detected differences were not consistently significant, the data obtained for *Delosperma* indicate that elevated temperatures stimulated the formation of flower buds in the investigated genotype (Fig. 3). In addition, a promotion of plant growth at the higher temperature level was prominent particularly in *Delosperma* (Fig. 4A). Despite the relatively small differences between regimes T1 and T2 with regard to flower bud numbers, the nightly temperature drop appeared to have a negative impact on flower formation. The determination of aboveground fresh masses in future experiments could clarify whether the low night temperatures had an effect on plant growth as well. Due to its area of origin, *Delosperma* is most likely better adapted to growing and flowering periods during summer. In Central Europe, some *Delosperma* species and cultivars (e.g. the `Jewel of desert´ series by FlorSAIKA) are currently used as bedding plants in rock gardens, particularly because they tolerate frost (Van Jaarsveld and De Pienaar, 2004).

Also other studies on flower induction in South African plant species revealed temperature as the most important factor and in the majority of conducted experiments, lower temperature levels were found to be promotive. Van Rooyen at al. (1991) detected no photoperiodic impact for the annual species *Dimorphoteca sinuata*, *Ursinia calenduliflora* (Asteraceae) and *Heliophila pendula* (Brassicaceae), but observed that flower primordia were formed faster at lower temperatures (in a range from 12 °C to 27 °C). However, also in these plants the time span between flower induction and anthesis was shorter at higher temperatures. Adams et al. (1998) obtained very similar results during investigations of *Osteospermum jucundum* ("Cape Daisy", Asteraceae). The optimal temperature for flower induction in this species was 10.6 °C and the subsequent development of inflorescences was fastest at 23.5 °C.

Most studies on flower induction and development of South African plant species with a horticultural background focused on geophytes. Ehrich et al. (2009, 2010) investigated different Iridaceae species and observed that dormancy of corms could be maintained at temperatures above 20 °C. Flower induction was achieved by decreasing night temperatures to a maximum of 13 °C. Ehrich et al. (2010) furthermore pointed out that low light intensity can as well cause the abortion of flower buds and that

assimilation lighting is required for the production of plants with adequate quality under Central European conditions. Thompson et al. (2011) detected an obligate cold requirement for the formation of flower buds in one of three investigated *Watsonia* (Iridaceae) species. In none of the species, photoperiod was an obligate factor, but day lengths of at least 12 hours had a positive impact on flower formation. After induction, flower buds discontinued their development when temperatures exceeded 25 °C.

Generally, temperature is one of the well-known flower inducing factors. However, the effects of a prolonged period of cold (vernalization) and those of the ambient growth temperature should be distinguished (Srikanth and Schmid, 2011). The requirement of a vernalization period to form flowers in the subsequent growing season and the molecular background of this pathway has been intensively studied in *Arabidopsis thaliana*. In this plant, floral repressors (e.g. *FLOWERING LOCUS C*; *FLC*) are epigenetically silenced in response to vernalization (Srikanth and Schmid, 2011). Whether similar physiological reactions regulate flowering in the investigated *Lampranthus* genotypes and other South African plant species that prefer cooler temperatures remains speculative, but screening for orthologues of known vernalization-related genes could provide new evidence.

In the annual Aizoaceae species *Mesembryanthemum crystallinum*, flowering starts after reaching a certain fresh mass under appropriate culture conditions, but any kind of stress (e.g. drought, salt) accelerates the onset of bud formation (Adams et al., 1998). Struck (1994) observed higher flower numbers during moist years in most of the studied Namaqualand plant taxa, but Aizoaceae species flowered more prolifically in times with low precipitation. Future experiments should therefore verify possible impacts of water availability on flower numbers and plant quality.

Conclusions

None of the investigated midday flowers showed an obligate photoperiodic response, although flowering in their natural areas of distribution either takes place during short winter days (*Cephalophyllum* and *Lampranthus*) or during summer (*Delosperma*). The experiment furthermore indicated that high daily light integrals are required to obtain an adequate plant quality.

In contrast to the plant community of the northern hemisphere, many South African species have their vegetation period during winter when temperature and precipitation patterns are favorable. In *Lampranthus*, mean daily temperatures of 14 °C stimulated the formation of flower buds (Fig. 3), whereas 20 °C repressed flowering almost completely in one genotype. As in several other plants from South Africa (see above), temperature appears to be the most crucial factor for flower induction in this genus. Given that other *Lampranthus* species and cultivars exhibit a similar flowering phenology, Central European cultivators aiming at offering these plants in full bloom during summer could include a chilling period in their production cycle.

Cephalophyllum, a genus which occurs in similar climates as *Lampranthus*, was not affected by temperature, although 20 °C (regime T3) presumably exceeded the mean temperatures during its flowering period at the South African Cape.

The observed stimulation of flower formation and plant growth at higher temperatures in the investigated *Delosperma* genotype (Fig. 3) is in line with the genus´ adaption to summer rainfall climates. For this reason, *Delosperma* cultivars are well-suited items for the sales period of garden plants in Central Europe.

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Table 1. Mean numbers of open flowers per plant documented during nine weeks at different photoperiods and 20 °C mean daily temperature (flower buds were observed in all genotypes and treatments); ± Standard deviation, NI = night interruption

Genotype	Regime P1 long-day	Regime P2 short-day	Regime P3 short-day (+ 2h NI)
Delosperma I	39.8 ± 2.3	27.0 ± 2.9	21.3 ± 2.9
Cephalophyllum I	3.7 ± 0.4	4.0 ± 1.3	2.3 ± 0.5
Lampranthus I	0.6 ± 0.4	0.2 ± 0.3	0.6 ± 0.1
Lampranthus II	0.5 ± 0.3	0.0	0.0

Figure 1. Flower portraits of the investigated genotypes; A) *Delosperma* I, B) *Delosperma* II, C) *Cephalophyllum* I, D) *Cephalophyllum* II, E) *Lampranthus* I, F) *Lampranthus* II

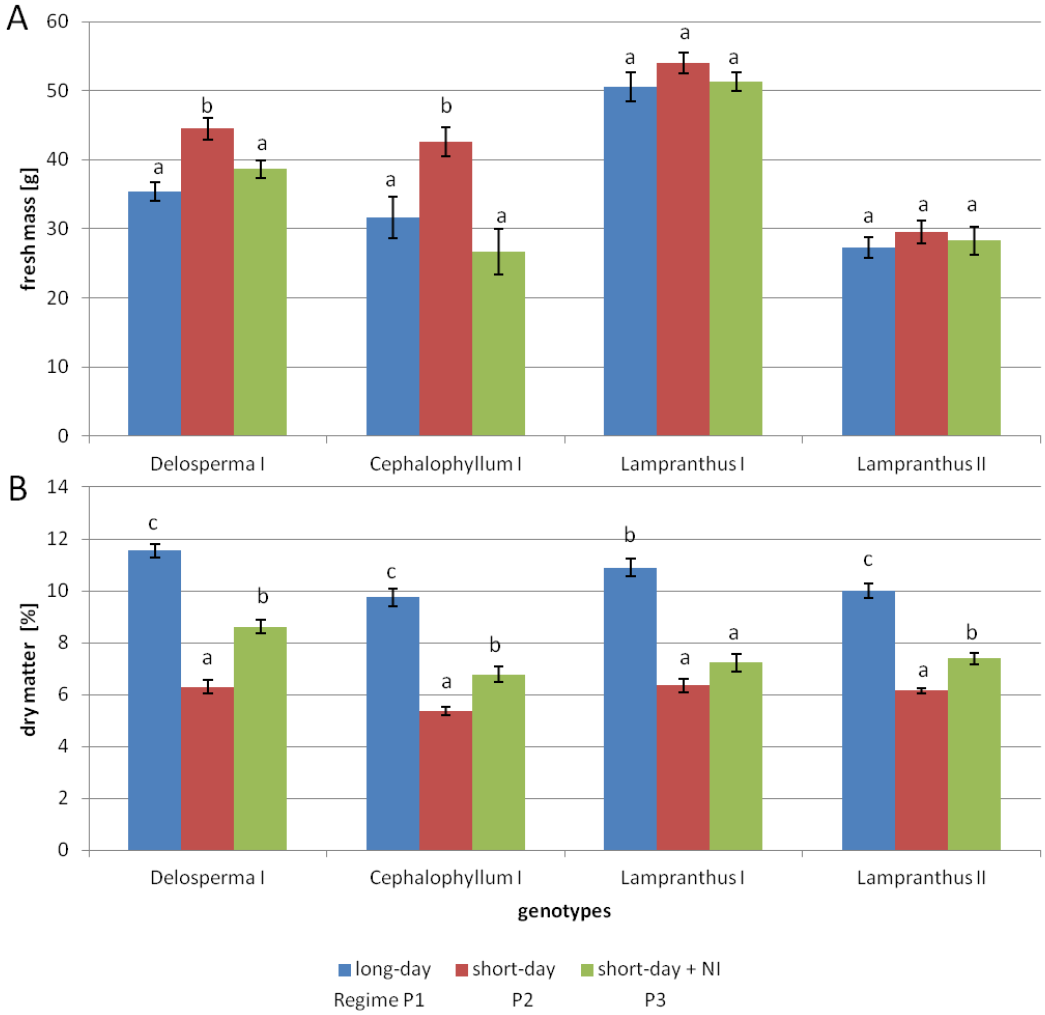

Figure 2. Plant aboveground fresh masses (A) and dry matter contents (B) determined after nine weeks cultivation at different photoperiods; NI = night interruption, I = Standard deviation, Tukey test ($\alpha = 0.05$), different letters indicate statistically significant differences.

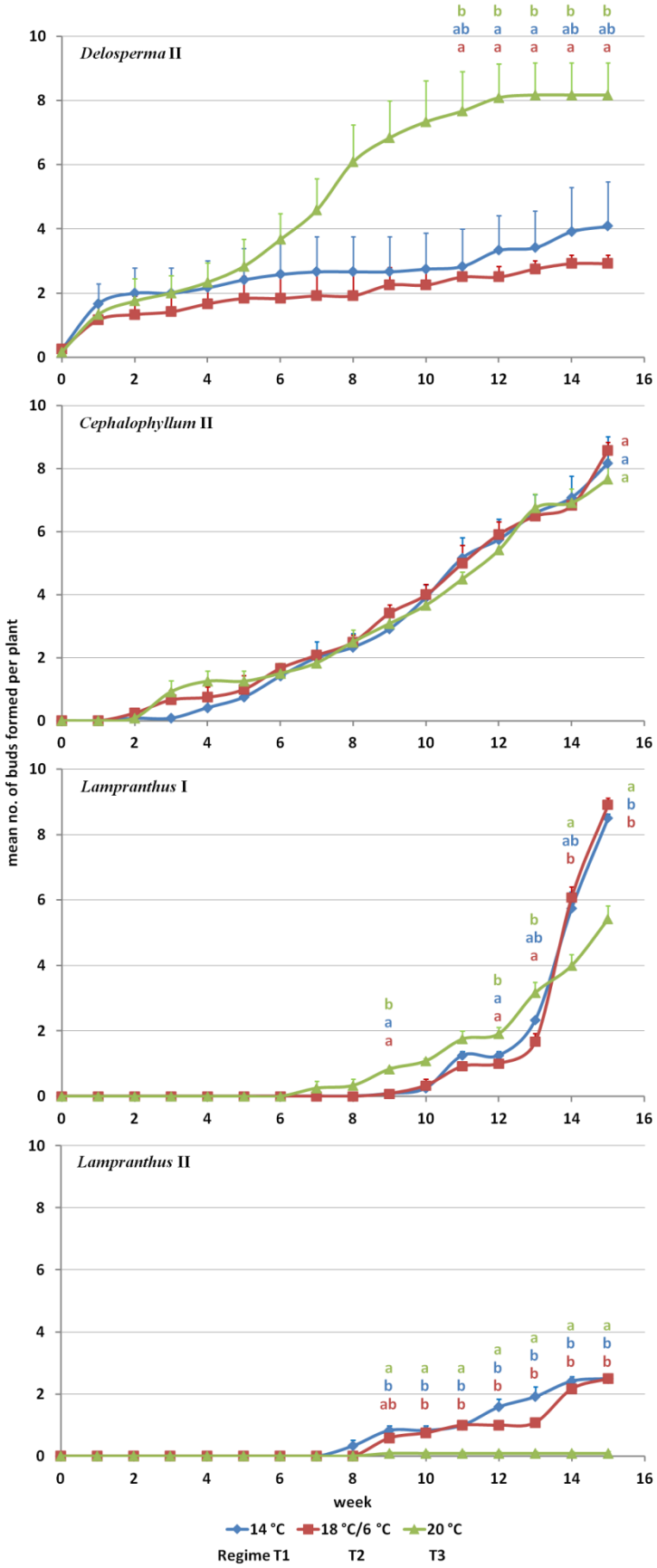

Figure 3. Mean numbers of flower buds formed per plant during 15 weeks at different temperature regimes; I = ½ x Standard deviation; different letters indicate statistically significant differences between treatments (Tukey test; $\alpha = 0.05$).

Figure 4. Representative plants cultivated under temperature regimes 1 (14 °C), 2 (18 °C/6 °C) and 3 (20 °C; from left to right) after 13 weeks; A) *Delosperma* II, B) *Cephalophyllum* II, C) *Lampranthus* I, D) *Lampranthus* II; arrows indicate unopened flower buds in the *Lampranthus* genotypes

Chapter 3: Manuscript II

Localization and overcoming of hybridization barriers in *Delosperma* and *Lampranthus* (Aizoaceae)

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Type of article:	Research article
Share of work:	80 %
Contribution to the manuscript:	planned and performed all experiments, analyzed data, prepared all figures and wrote the manuscript The results of pollen tube growth analyses by aniline blue staining as well as some data concerning pollen viability, seed set and seed germination were obtained in previous experiments related to the Master thesis "Untersuchungen zur Blütenbiologie und Samenentwicklung bei verschiedenen Vertretern der *Aizoaceae* (Mittagsblumengewächse)" (Braun, 2012)
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Localization and overcoming of hybridization barriers in *Delosperma* and *Lampranthus* (Aizoaceae)

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Abstract The large succulent plant family Aizoaceae offers an enormous diversity of growth forms and flower pigmentations as well as prevalent tolerance to drought. Although many family members have potential for a wider use as ornamental plants, directed breeding of midday flowers is still in its infancy and only few cultivars have been launched onto the market so far. Possibly, this fact can be ascribed to the presence of cross-incompatibilities. The present study aimed at localization of intra- and intergeneric hybridization barriers between different genotypes of the genera *Delosperma* and *Lampranthus* using various methods. Aside from distinct self-incompatibility, the analyses revealed that pollen tube growth towards the ovules was not inhibited, even after intergeneric pollinations. While cross-combinations within *Delosperma* appeared to be compatible to a high extent, hybridizations of the investigated *Lampranthus* genotypes and intergeneric combinations were largely unsuccessful. Investigations of embryo sacs following wide crosses using ovule clearing and differential interference contrast microscopy revealed fertilization failures as well as abnormal early embryogenesis. Seedlings, which were anyhow obtained after wide crossings, mostly exhibited partial or complete loss of chlorophyll causing frequent hybrid breakdown. We therefore conclude that hybridization barriers between the investigated midday flowers occur both pre- and postzygotically.

Keywords Albinism • *Delosperma* • Embryogenesis • *Lampranthus* • Pollen tube guidance • Reproductive isolation

Introduction

The Aizoaceae, whose origin and main area of distribution are located in the southern African Greater Cape Floristic Region (GCFR), is a hyperdiverse family of succulent plants. It is composed of four subfamilies (Sesuvioideae, Aizoideae, Mesembryanthemoideae and Ruschioideae) and comprises a higher number of taxa (~ 1830) than all other succulent families including Cactaceae (Klak et al. 2003). Approximately 1600 midday flower species belong to the large Ruschiae tribe (subfamily Ruschioideae) (Klak et al. 2013), which was identified as the most rapidly diversifying known group of plants (Klak et al. 2004). Recent molecular approaches revealed that the origin of most core ruschioids dates back only ~ 1.5-3.31 million years (Valente et al. 2014). The evolution of new taxa on the one hand often originates in interspecific hybridization events (Abbott 1992), but on the other hand also necessitates formation of reproductive barriers, which ensure genetic isolation. Depending on whether such isolating mechanisms act before or after fertilization, commonly two different categories are distinguished: (1) Prezygotic hybridization barriers include spatial or temporal separation, pollinator-specificity and specificity in pollen-pistil interactions. (2) Postzygotic hybridization barriers prevent the formation of seeds after fertilization or reduce the viability and/or fertility of the hybrid progeny (Rieseberg and Carney 1998; Swanson et al. 2004). After comprehensive literature review, Linder (2003) postulated that the extremely high diversity, which can also be found in other families of the Cape flora, is based on diverse limitations to gene flow, but pointed out that almost no experimental approaches on sexual incompatibilities between species from this area have been conducted. Interspecific hybridizations in midday flowers were intensively studied in the invasive genus *Carpobrotus* (Albert et al. 1997; Gallagher et al. 1997; Vilà and D´Antonio 1998; Vilà et al. 1998; Suehs et al. 2004; Suehs et al. 2006), whereas the number of

documented naturally emerged hybrids among other Aizoaceae taxa is low. However, spontaneous intergeneric hybridizations, such as *Lapidaria* x *Lithops* or *Gibbaeum* x *Muiria* were observed in a few instances (Hammer and Liede 1990, 1991).

Many family members are attractive ornamental plants, particularly because of their intensively coloured flowers and their tolerance to drought. Although horticulturalists for a long time carried out wide crosses within the Aizoaceae, only a negligible number of stable hybrids resulted from their efforts (e.g. Poindexter 1931; Brown 1920; Schwantes 1954; Hammer and Liede 1990, 1991; Hammer 1995) and, to our knowledge, not a single one contributed to the development of commercially traded cultivars. Hammer and Liede (1990, 1991) and Hammer (1995) performed extensive cross breeding experiments in midday flowers and observed diverse expressions of incompatibility, such as dieback of pollinated flowers, formation of empty seed capsules or non-viable seeds, albinism in seedlings and low hybrid vigor. Hammer and Liede (1990) furthermore postulated that hybridization barriers between species from geographically separated habitats are weaker than between those sharing the same areas of distribution.

The present study aims at characterization and overcoming of hybridization barriers within and between the genera *Delosperma* and *Lampranthus*, whose taxa are already in use as ornamental plants. The natural habitats of both selected Ruschiae genera are spatially separated for the most part. *Lampranthus* almost exclusively occurs in winter rainfall areas near the Cape of Good Hope, whereas *Delosperma* is widely distributed in summer rainfall regions of southern Africa (Hartmann 2001). The analyses presented in this study contribute to a better understanding of reproductive isolation mechanisms in the Ruschioideae and pave the way for the development of new midday flower cultivars.

Materials and methods

Plant materials and greenhouse cultivation

Investigations were carried out on six genotypes of the genus *Delosperma* (referred to as *D.* genotypes I-VI below), on five genotypes of the genus *Lampranthus* (*L.* genotypes I-V; Fig. 1) and on hybrids which resulted from cross-pollinations between these plants. Because of their horticulturally attractive traits (regarding flower pigmentation and/or growth form) all genotypes were previously considered as adequate starting material for a commercial breeding program, but detailed information about the taxonomic background or breeding history was not available. However, *Lampranthus* genotype I shared growth and flower characteristics with the species *L. roseus*, *L.* genotype III with *L. aureus*, and *L.* genotype IV with *L. bicolor* (e.g. described by Van Jaarsveld and De Pienaar 2004). The *Lampranthus* genotypes II and V appeared to be morphologically identical. Two of the investigated *Delosperma* genotypes are cultivars developed by the Japanese breeder Koichiro Nishikawa (V, `Ruby´; VI, `Garnet´).

The genome sizes of most plants involved in the present study were previously determined flow cytometrically and ranged between 1.18 and 2.36 pg/2C (*Delosperma* genotype **I**: 1.18 pg/2C; *D.* **V**: 1.27 pg/2C; *Lampranthus* genotype **I**: 2.36 pg/2C; *L.* **II**: 1.95 pg/2C; *L.* **III**: 1.6 pg/2C; *L.* **IV**: 1.94 pg/2C). Furthermore, *Lampranthus* genotype I was found to be diploid ($2n=2x=18$) (Braun and Winkelmann, submitted manuscript).

The plants were propagated by cuttings and cultivated in peat/sand substrate (3:1) at 20 °C mean daily temperature. Relative humidity in the greenhouse was adjusted to 40-60 % by automatic ventilation. High pressure sodium lamps (Philips MASTER Agro 400 W) were used to ensure minimum daily photoperiods of 16 hours and for supplementary lighting when solar radiation fell below 25 klx. The plants were irrigated manually and fertilized once a week with 0.1 % Hakaphos rot© (8 % N: 12 % P_2O_5: 24 % K_2O, Compo, Münster, Germany).

Pollen viability tests

Pollen of five to fifteen completely opened flowers of each parental genotype (except for *Lampranthus* genotype III) and of those hybrids, in which flower formation was observed, was collected in Petri dishes and mixed with

a needle. Afterwards small amounts of pollen were transferred to three Petri dishes, respectively, containing Brewbaker and Kwack (1963) germination medium (solidified with 5 g/l Gelrite©) with a brush. The inoculated dishes were sealed with Parafilm© (Bemis Company, Neenah, USA) and placed at room temperature under ambient light for 24 hours. Afterwards, at least 300 pollen grains in each Petri dish were evaluated light microscopically (AxioScope A1, Zeiss, Germany). All grains which had developed a pollen tube that was longer than their diameter were regarded as viable. Mean germination rates were averaged over the results of the three technical repetitions with the exception of putative *Lampranthus* hybrids in which germination tests were performed at three different dates, due to limited availability of flowers. For the same reason, pollen of the intergeneric hybrid *Delosperma* III x *Lampranthus* I (b) was tested without repetition.

Analyses of pollen tube growth *in situ*

Pollen germination and tube growth *in situ* were evaluated between January and October 2011 following self-pollinations as well as after intra- and intergeneric cross-pollinations among *Delosperma* genotypes I-IV and *Lampranthus* genotypes I-IV. Flowers selected for pollination were emasculated before anther dehiscence in a late bud stage by opening flower buds with a scalpel and removing the male organs by forceps. After each emasculation, tools were cleaned with 70 % ethanol (v/v) to avoid unintended transfer of pollen grains. Pollinations were carried out by dipping open flowers of the paternal partners on the completely unfolded stylodia of the maternal flowers two to three days after emasculations. Five pollinated flowers of each combination were harvested 4, 24, 48, 72 and 120 hours after pollination, respectively, and fixed in ethanol (99 %)/lactic acid (90 %) (2:1). Before staining, the fixed ovaries were rinsed in deionized water and macerated in 1 M NaOH at 60 °C (*Delosperma* 30 minutes, *Lampranthus* 60 minutes). The macerated ovaries were rinsed a second time and afterwards immersed in aniline blue staining solution (100 mg aniline blue and 768 mg $K_3PO_4H_2O$, dissolved in 100 ml deionized H_2O) for at least 24 hours. Directly before the microscopic analyses, stained ovaries were either squashed completely on microscope slides or cut in thin slices using razor blades. The presence of pollen tubes was examined on stigma surfaces, in transmitting tracts and in the ovary by fluorescence microscopy (Zeiss filter set Fs02, emission: 370 nm, excitation: 509 nm).

Observations of fertilization and early embryogenesis

Observations of embryo sac contents were carried out on cleared ovules following self-pollinations as well as after intra- and intergeneric cross-pollinations among *Delosperma* genotypes I, IV and VI and *Lampranthus* genotypes I, II and IV. Emasculations and pollinations were performed for 30 combinations as described above between July 2013 and July 2015. Five ovaries of each combination were harvested and fixed five and ten days after pollinations, respectively. Ovaries were bisected using razor blades and containing ovules were transferred on microscope slides with two cavities (Knittel Gläser, Braunschweig, Germany). Afterwards the ovules in each cavity were covered with 80-95 µl Hoyer´s solution with the following composition: 50 g chloral hydrate, 3.5 g gum arabic (Sigma-Aldrich, St. Louis, USA) and 2.5 g glycerine (Riedel-de Haën, Seelze, Germany) dissolved in 20 ml deionized water. The cavities were sealed with cover slips and nail polish and the samples were placed at room temperature for at least two days to bleach. The microscopic analyses of cleared ovules were performed using a Zeiss AxioScope A1 equipped with Nomarski (differential interference contrast= DIC) prisms and filters. Observations focused on pollen tube remnants in micropyles, embryo sac expansion and embryo development.

Evaluation of seed set and seed germinability

Whenever sufficient numbers of flowers were available, further cross- and self-pollinations were performed to observe the maturation or abortion of seed capsules, to determine and compare the extent of seed sets and to verify the germinability of resulting seeds. Capsules were harvested when the respective pedicel became lignified and were afterwards stored at room temperature. Using a stereo microscope, mature seeds of each capsule were counted and separated from unfertilized ovules, dried funiculi and obviously hollow seed coats. Depending on their availability, 1 to 414 seeds (obtained from one to eight capsules) of each combination were utilized for *in vitro* germinability tests. Seeds were surface sterilized in 1 % NaOCl solution (supplemented with a drop of Tween 20) for two minutes and rinsed three times (1, 2 and 5 minutes) in sterile deionised water. Using forceps the seeds were transferred to Petri dishes containing half strength Murashige and Skoog (1962) medium with 10 g/l sucrose, which was solidified with 3 g/l Gelrite© (Duchefa, Haarlem, Netherlands). The pH value of

the medium was adjusted to 5.8 using 0.1 M KOH. The Petri dishes were sealed with Parafilm© (Bemis Company, Neenah, USA) and placed at 23 °C and daily photoperiods of 16 hours (40 µmol $m^{-2}s^{-1}$ PPFD). For a duration of eight weeks the Petri dishes were checked weekly for seed germinations, and viable seedlings were transferred to larger vessels containing full strength MS medium (30 g/l sucrose, 3 g/l Gelrite©, pH 5.8).

Embryo rescue (ovule culture)

Ovule cultures were conducted in two approaches following cross-pollinations, which were performed as described above. For the first approach (2011) two to five swollen ovaries (depending on the availability of receptive flowers) were harvested three, four, five and six weeks after pollination (WAP), respectively. The ovaries were dipped in 70 % (v/v) ethanol for 30 seconds and afterwards surface sterilized in 2 % NaOCl solution (supplemented with Tween 20). After three times rinsing (1, 2 and 5 minutes) in sterile deionized water, the ovaries were quartered using scalpels and containing ovules were transferred in equal shares to Petri dishes containing two different media compositions. Both media were composed of MS salts in half strength (Murashige and Skoog 1962), 680 µM Glutamine and 3 g/l Gelrite© (pH 5.8), but differed in regard to sucrose concentrations. Medium 1 contained 1 % and medium 2 2.5 % (w/v) sucrose. The Petri dishes were sealed with Parafilm© and placed at 23 °C in darkness for four weeks. After that time, the ovules were exposed to light (40 µmol $m^{-2}s^{-1}$ PPFD) and germination was evaluated ten weeks after ovary harvest.

For the second approach (2014) ovaries were processed four, five, six and seven weeks after cross-pollinations. This time, containing ovules were evenly transferred to three different media (basic composition see above; no Glutamine). Media 1 and 2 contained 3 % (w/v) sucrose and medium 2 was supplemented with 0.5 mg/l Zeatin. The sucrose concentration of medium 3 was 6 % (w/v). The Petri dishes were kept in darkness until 60 DAP and were afterwards exposed to light (40 µmol $m^{-2}s^{-1}$ PPFD). Ovules were transferred to fresh medium six weeks after exposure to light, respectively. Germinations were evaluated during a time span of eight weeks.

AFLP analysis

Of each genotype, approximately 100-150 mg fresh and young leaf material (leaf length: 2-5 mm) were immersed in liquid nitrogen and pulverized. In accordance with the CTAB method established by Doyle and Doyle (1987), DNA was extracted from the homogenates as described below. Five hundred µl of CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 2 % w/v CTAB, 20 mM Na_2EDTA, 1.4 M NaCl, 3 % w/v PVP, 1 % v/v β-mercaptoethanol) and RNAse A (35 U) were added to each sample in 2 ml reaction tubes. After incubation at 65 °C for one hour, the samples were homogenized with 500 µl chloroform: isoamyl alcohol (24:1) for 20 minutes and centrifuged at 6000 x g for 25 minutes. The supernatant was mixed with another 500 µl chloroform: isoamyl alcohol (24:1) and centrifuged for a second time (6000 x g, 25 minutes). Subsequently, 1 ml ice-cold isopropanol was added to the supernatant and samples were left for DNA precipitation at room temperature for two hours. In a next step, samples were centrifuged at 6000 x g for five minutes to obtain DNA pellets. The pellets were washed in 96 % (v/v) and 70 % (v/v) ethanol, air-dried and afterwards dissolved in 200 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). DNA yield and purity were checked photometrically (NanoDrop 2000, Thermo Scientific, Waltham, USA) and DNA integrity was tested by agarose (1 %) gel electrophoresis.

AFLP analyses were performed according to Vos et al. (1995) with minor modifications. Approximately 250 ng DNA of each genotype were digested with the restriction enzymes *Hind*III (10 U) and *Mse*I (3 U) at 37 °C over night. *Hind*III (5´-AGCTGGTACGCAGTCTAC) (2.5 pmol) and *Mse*I (5´-ACTCAGGACTCAT) (25 pmol) adapters were ligated to the restriction fragments with T_4 ligase (0.25 U) at 37 °C for 3.5 hours. Pre-amplifications were performed using *Hind*III and *Mse*I primers without selective nucleotides and PCR products were subsequently checked by agarose (1.5 %) gel electrophoresis. Selective amplifications were performed using *Hind*III primers (IRD700 or 800 labeled at the 5`-end) and *Mse*I primers with three selective nucleotides at the 3`-end (primer combinations *Hind*III-ATT/*Mse*I-GGC, *Hind*III-ATA/*Mse*I-GAC, *Hind*III-CAT/*Mse*I-GAC, *Hind*III-CAT/*Mse*I-GTA). PCR fragments were separated and analyzed in 6 % denaturing polyacrylamide gels in a LI-COR DNA sequencer (LI-COR Global IR2 4200LI-1 Sequencing System, Lincoln, USA). The obtained banding patterns were scored manually and transformed into a I/0 matrix. DNA fragments present in the paternal and progeny matrix, but absent in the maternal pattern were assumed to confirm the status of hybridity. Calculations of genetic distances using the Jaccard similarity index and a cluster analysis were performed with

the FAMD software package 1.31 (http://www.famd.me.uk/famd.html). After a bootstrap analysis (Felsenstein 1985) with 1000 replicates, the obtained data were used to create a Neighbor-Joining dendrogram, which was visualized with the TreeView software (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

Flower development and morphology

Both genera produce actinomorphic hermaphroditic flowers with inferior ovaries, which are divided into five loculi. The pigmented flower leaves are petaloid staminodes (due to an alternative flower organ identity program; Brockington et al 2012), which surround an androeceum consisting of dozens of stamens. All genotypes exhibited distinct proterandry. Anther dehiscence and pollen dispersal started at the beginning of anthesis. At this stage stamens were congregated in the center of flowers and covered the subjacent, only rudimentarily developed stigma. The filaments bent outwards during anthesis and thereby opened up access to five papillous stylodia, which simultaneously unfolded completely and distinctly gained in size. The stylodia united basally forming a relatively short pollen tube transmitting tissue, which terminated in the ovary. Each ovary locule contained numerous anatropous to campylotropous ovules, which were connected to parietal papillous placentas *via* long funicles. Compatible pollinations induced swelling of ovaries, which afterwards ripened to hydrochastic seed capsules (open after thorough moistening) over a period of several weeks (*Delosperma*: 4-10 weeks, *Lampranthus*: 8-12 weeks). Readers interested in further details of flower morphology in Ruschioideae species might consult the outstanding descriptions of Ihlenfeldt (1960) and Volgin (1998).

Pollen viability

The *Delosperma* genotypes shed pollen with high germination rates between 58 % (*D.* genotype VI; Table 1) and 73 % (*D.* genotypes III and V), except for *D.* genotype IV, of which only 9 % of the examined pollen grains germinated. Additional microscopic observations revealed that *D.* genotype IV released notable portions of malformed as well as very small grains. The *Delosperma* hybrid IV x V (obtained *via* ovule culture; Fig. S1, Online Resource 1) released pollen with a germination rate of 8 %. Pollen of the intergeneric hybrid *D.* III x *L.* I (b) (Fig. 5 d-f) had a germination rate of 56 %. The pollen germination rates in parental *Lampranthus* genotypes ranged between 58 % (*L.* genotype II) and 87 % (*L.* genotype IV). The tested intrageneric *Lampranthus* hybrids showed distinctly lower pollen germination rates (8-17 %) and the grain diameters appeared to be extremely variable (Fig. S2, Online Resource 1). In all examined samples relatively long tubes (~ 100 - > 2000 µm) had developed from the majority of germinated pollen grains and evident growth abnormalities or tube burst were not observed.

Pollen tube growth *in situ*

An overview of pollen tube growth results obtained in 34 self- and cross-combinations is given in Table 2. Following self-pollinations of *Delosperma* genotypes I-IV pollen grains germinated on the stigma surfaces, but the pollen tubes only reached short lengths and did not penetrate the stigmatic papillae. Consequently, no pollen tubes were observed in transmitting tracts and ovaries. Although a numerical quantification was not possible, only low portions of germinated pollen grains were detected on self-pollinated stigmas of *Lampranthus* genotypes I-IV. In contrast to *Delosperma,* in self-pollinations of *Lampranthus* pollen tubes grew through the transmitting tract towards the ovary and in *L.* genotypes I and IV some pollen tubes were observed to penetrate the micropyles of ovules. No pollen tubes were found in ovaries of *L.* genotype II after aniline blue staining.

Four hours after intrageneric cross-pollinations of *Delosperma* and *Lampranthus* genotypes high numbers of pollen grains had germinated on the stigmas and bundles of pollen tubes had reached the transmitting tissue (Fig. 2a). In *Delosperma* combinations, first pollen tubes were observed to penetrate micropyles of ovules 24 hours

after pollination. In the distinctly larger *Lampranthus* flowers, pollen tubes were observed in the proximity of ovules 48 hours after pollination (Fig. 2c). Ovaries harvested 120 hours after intrageneric cross-pollinations were visibly swollen in both genera.

Also in each of the tested intergeneric combinations, pollen tubes were detected in micropyles of ovules 24 to 48 hours after pollination (Fig. 2d). Again, the majority of ovaries harvested 120 hours after intergeneric cross-pollinations were swollen and some of the ovules, which were attached to a pollen tube, had increased in size.

Fertilization and early embryogenesis

Following self-pollinations of *Delosperma* genotypes I, V and VI neither pollen tube remnants nor embryonic tissues were observed in cleared ovules. Ten days after self-pollinations ovules were not swollen, but partly malformed. Where identifiable, embryo sacs had retained their circular shape. Basically the same was also observed after self-pollinations of *Lampranthus* genotype I. In contrast, ovules excised from flowers of *L.* genotypes II and IV were in part attached to pollen tube remnants. The embryo sacs within most of these ovules appeared not to be expanded and contained no embryonic structures. Nevertheless, a few pre-globular embryos were observed in two flowers of *L.* genotype II five days after self-pollination (Table 3).

Following all cross-pollinations performed within the genus *Delosperma* pollen tube remnants were found in micropyles of most ovules. Embryo sacs within these ovules distinctly expanded in length while those in ovules which were not penetrated by pollen tubes retained their original circular shape. Furthermore, embryonic structures were detected in most ovules showing expanded embryo sacs. Pre-globular embryos were observed five days after *Delosperma* cross-pollinations, while the typical globular stage was reached after ten days (Fig. 3a). In several fertilized ovules formation of nuclear endosperm was visible at the micropylar poles of embryo sacs. Five days after the intrageneric *Lampranthus* combinations pollen tube remnants and expanded embryo sacs were observed in the majority of excised ovules. At this point in time zygotes and rudimentarily developed pre-globular embryos were visible at the micropylar poles. Ten DAP embryos had further developed, but had not reached the globular stage yet (Fig. 3b).

Embryo sac expansion and swelling of ovules were only rarely observed after intergeneric crossings of *Delosperma* and *Lampranthus* genotypes, although numerous ovules were penetrated by pollen tubes in the majority of examined ovaries. Cross-pollinations, in which *Lampranthus* genotype I was combined with *Delosperma* genotype VI, did not result in embryo development (Table 3). However, when *D.* genotypes I, IV and V were used as pollen donors expanding zygotes, abnormally developed embryos (Fig. 4c) and, rarely, pre-globular embryos were detected (Table 3). Following the combination *Lampranthus* I x *Delosperma* I several ovules were found to be penetrated by two pollen tubes (Fig. 4a). Moreover, in a few embryo sacs pollen tubes had not stopped growing at the synergids and curled within the female gametophyte (Fig. 4b). Also in the combinations *Lampranthus* II x *Delosperma* I and *L.* IV x *D.* I most ovules within the ovaries were unfertilized, but a few apparently normally developed pre-globular embryos as well as only rudimentarily developed embryonic structures were found. Ten days following pollinations of *Delosperma* flowers with *Lampranthus* pollen many ovules were malformed and shrunken. Nevertheless, throughout all *Delosperma* x *Lampranthus* combinations fertilized ovules often contained aberrantly developed embryos (Fig. 4d, e) and, in rare cases, typical pre-globular and globular embryos.

Capsule maturation and seed formation

In both genera, self-pollinations did not result in capsule formation (Tab. 4). After self-pollinations of *Lampranthus* I, ovaries began to swell, but subsequently dried off within a couple of days. *Delosperma* flowers showed no visible reaction when self-pollinated.

Intrageneric cross-pollinations, in which *Lampranthus* I and *L.* II were used as seed parents, frequently induced capsule formation (67-96 % in *L.* I; 86-100 % in *L.* II). Following pollinations of *Lampranthus* I with pollen of five different *Delosperma* genotypes, many flowers dried off within a few days. However, 9-40 % of pollinated flowers completed the transformation to woody seed capsules. After the cross-combination *Lampranthus* II x

Delosperma I flower abortion was not observed. The time required for capsule maturation appeared to be variable and took eight to twelve weeks in both *Lampranthus* genotypes.

The rates of capsule formation after intrageneric *Delosperma* crossings varied and ranged, except for those in which *D.* IV was pollinated, between 15 and 100 % (Table 4). Pollinated flowers either dried off relatively fast or showed a distinct increase in ovary size within the first week. Swelling ovaries of *Delosperma* genotypes I-III, V and VI further developed to seed capsules during four to ten weeks. Although pollinations induced initial ovary swelling also in *Delosperma* IV, capsule formation was not observed in this genotype.

Intergeneric pollinations, in which *Delosperma* genotypes were used as seed parents, were unsuccessful in most cases. Four capsules were formed after combination *Delosperma* I x *Lampranthus* I (13 %) and one capsule after combination *D.* III x *L.* I (9 %, Table 4).

The mean numbers of completely developed seeds per capsule varied greatly between and within different cross-combinations (Tab. 4). For example, the average number of seeds in 26 capsules formed after the combination *L.* I x *L.* II was 32, whereas the actual numbers ranged between three and 80 seeds per capsule. Further intrageneric combinations of this seed parent with *L.* III and *L.* IV resulted in mean numbers of two and eleven seeds per capsule, respectively. Seed capsules of *Lampranthus* I, which have formed after intergeneric crossings contained five to 14 seeds on average.

The cross-combination of *Lampranthus* I and II resulted in relatively high numbers of seeds, also when *L.* II was used as maternal partner (Tab. 4). Again, the actual numbers of seeds varied greatly (min. 3 – max. 90). In contrast, capsules formed after the combinations *L.* II x *L.* III, *L.* II x *L.* IV and *L.* II x *D.* I were generally almost empty (Tab. 4).

All mature capsules, which have developed following intrageneric *Delosperma* combinations contained seeds. The highest number of seeds (227) was found in a capsule of *Delosperma* III after crossing with *D.* II. The average number of seeds in this combination was 150. While after most of the other *Delosperma* crossings average seed numbers between 45 and 132 were detected, only 21 to 24 were found after combinations in which *D.* IV was used as pollen parent. Each *Delosperma* genotype had been pollinated with pollen of *Lampranthus* I, but capsules have formed only in two of six combinations. These turned out to contain only three to four seeds (Tab. 4).

Seed germinability and seedling appearance

In both genera, seed germinations were already observed within the first week after transfer to culture medium. However, while the majority of *Delosperma* seeds germinated during the first four weeks, germinations within *Lampranthus* mostly occurred after week four. No further germinations were observed after eight weeks. Germination rates of seeds which resulted from intrageneric hybridizations ranged between 2 and 53 % in *Lampranthus* and between 11 and 94 % in *Delosperma* (Tab. 4). The cotyledons and primary leaves of intrageneric *Lampranthus* seedlings were pale green (Tab. 4) and only grew very slowly. Within *Lampranthus*, solely plantlets which resulted from the cross-combinations *Lampranthus* I x II and *L.* I x V developed normally and of those only six (referred to as *L.* I x II (a)-(e) and *L.* I x V below) survived acclimatization to greenhouse conditions. Chlorophyll deficiency and chlorotic leaf tips remained during the adult developmental stage of these plants (Fig. 5a and b). The hybrids *L.* I x II (a), (c), (d) and (e) showed intermediate leaf morphology (Fig. 5b), while the appearance of *L.* I x II (b) (Fig. 5a) and *L.* I x V neither corresponded to the maternal nor to the paternal parent. Flowers were only formed by the hybrids *L.* I x II (a), (c) and (d). Their flowers exhibited stamina and staminodes, which were very similar to those of the pollen parent. Intergeneric seedlings of *Lampranthus* I contained no chlorophyll at all (Tab. 4, Fig. 5c) and stopped growing shortly after germination. Leaf colour abnormalities or growth retardation were not observed in intrageneric *Delosperma* seedlings. Four seedlings, which resulted from the intergeneric combination *Delosperma* III x *Lampranthus* I had dark green leaves and two of them continued growing. These hybrids (referred to as *D.* III x *L.* I (a) and (b) below) largely resembled the maternal parent during their further development (Fig. 5d) and exhibited staminodes with mild violet pigmentation as only paternal trait (Fig. 5e). The only seedling obtained from the combination *D.* I x *L.* I was completely chlorotic (Tab. 4) and discontinued growing.

Embryo rescue (ovule culture)

Only low numbers of ovules germinated during the *in vitro* cultivation period and almost all emerged plantlets showed severe morphological abnormalities. As both ovule culture approaches remained largely unsuccessful and distinct impact of the different culture media did not become evident, the outcomes of different cross-pollinations with respect to time spans of *in planta* maturation are summarized in Table 5. All seedlings emerged from *Lampranthus* ovules did not exhibit the normal bipolar structure and often both, cotyledons and radicle were not distinctive (Fig. S3a,b, Online Resource 1). Plantlets, which were derived from intergeneric cross-pollinations, moreover lacked chlorophyll completely (Fig. S3c, Online Resource 1). None of these plants grew more than a few millimeters and progressive organ differentiation was observed in no case. Generally, germinations occurred more often from ovules, which were isolated after long-lasting *in planta* maturation (5-7 WAP; Table 5).

Also intrageneric cross-pollinations of *Delosperma* IV (a genotype, in which capsule formation was not observed; Table 4) mostly resulted in progeny exhibiting abnormal morphology and/or albinism (Fig. S3d, Online Resource 1). However, two viable plantlets emerged from the combination *Delosperma* IV x II, and one plantlet from the combination *D.* IV x V. Of these, only the latter survived acclimatization and formed first flowers after eight months of greenhouse cultivation (Fig. S1, Online Resource 1).

AFLP analysis

A total of 262 DNA fragments, of which only 30 were monomorphic for all investigated plants, were scored after AFLP analysis of six parental and ten progeny genotypes using four different primer combinations. The genetic relationships between the parental genotypes were visualized in a Neighbor-Joining tree (Fig. S4, Online Resource 1), which separated *Lampranthus* and *Delosperma* in two major clades. Hybridity was confirmed for all analyzed progeny genotypes, as banding patterns exclusively shared with the respective pollen parent were detected in the progeny patterns (examples given in Fig. 5f, Fig. 6 and Fig. S1b, Online Resource 1). Within the parental *Delosperma* genotypes, the lowest genetic distance was found between *Delosperma* III and V (0.54) and the highest between *Delosperma* IV and V (0.66, Table 6). Within *Lampranthus*, genetic distances ranged from 0.34 (genotypes II and V) to 0.42 (genotypes I and II). The minimum genetic distance between both genera was 0.73 (*Delosperma* IV and *Lampranthus* I) and the maximum distance was 0.83 (*Delosperma* III and *Lampranthus* I). All hybrids obtained from the intrageneric cross-pollinations showed more or less similar genetic distances to the seed and the pollen parent. In contrast, the two intergeneric hybrids obtained by the combination *Delosperma* III x *Lampranthus* I showed distinctly higher genetic distance to the pollen parent (0.76-0.77) than to the seed parent (0.32-0.38).

Discussion

The evaluation of capsule and seed development following hand-pollinations (Tab. 4) led to the general assumptions that all investigated *Delosperma* and *Lampranthus* specimen were self-sterile and that cross-pollinations within *Lampranthus* as well as intergeneric combinations relatively rarely resulted in hybrid progeny. In contrast, hybrid formation was easily obtained after cross-pollinations within *Delosperma*, even though some combinations appeared to be more successful than others. The conducted experimental approaches aimed at specifying the reasons for failure and success in greater detail and their outcomes are discussed in the following.

Lacking pollen viability was not the cause for hybridization failures

The pollen germination tests revealed that nearly all genotypes cross-pollinated for the present study shed viable pollen grains during anthesis. Ascertained germination rates of up to 87 % (*Lampranthus* genotype IV; Table 1) showed that (1) the nutrient medium adapted from Brewbaker and Kwack (1963) was suitable for testings of midday flower pollen and (2) that lacking pollen viability can be excluded as possible cause for absence of seed development. Like all other Aizoaceae species, the investigated plants form tricellular pollen grains (Prakash

1967a; Brewbaker 1967; Braun and Winkelmann 2015). Our results do not support the assumptions that tricellular pollen grains generally show low germination rates *in vitro* (Brewbaker and Kwack 1963; Daher et al. 2009). However, the pollen viability of *Delosperma* genotype IV, which possessed larger flowers and leaves than the other *Lampranthus* genotypes, repeatedly turned out to be rather low (9 %; Table 1). Whether this could explain the relatively low seed sets, which were obtained after using *D.* IV pollen in intrageneric hybridizations (Tab. 4), should be verified in future analyses. Aside from numerous malformed pollen grains, this genotype also shed grains with distinctly reduced diameters, in which nuclei could not be detected after fluorescence staining (not shown). Interestingly, almost identical observations were made in the Aizoaceae species *Aptenia cordifolia* (showing low pollen fertility; Pagliarini 1990) and within the Ruschioideae genus *Carpobrotus* (Suehs et al. 2006). Pagliarini (1990) detected that the formation of univalent chromosomes during meiotic diakinesis resulted in generation of micro nuclei and, subsequently, in sterile aneuploid pollen grains. Such meiotic aberrations could also explain the low viability and extremely varying grain diameters found in some hybrids originated from the present study (Fig. S2, Online Resource 1).

Self-incompatibility was observed in both genera, but with different phenotypes

Following self-pollinations of *Delosperma*, pollen germination, but no entrance of pollen tubes in stigmatic papillae was observed. Also in ovaries of *Delosperma* genotypes I, V and VI neither pollen tubes nor embryonic structures were detected in microscopic analyses (Table 3). These findings indicate that self-incompatibility in the aforementioned plants is most likely S-locus-controlled (see also Takayama and Isogai 2005). Although targeted analyses have only been rarely performed, midday flowers were often regarded as largely self-incompatible (e.g. Schwantes 1916, 1954; Brown 1920; van Jaarsveld and de Pienaar 2004). Mayer and Pufal (2007) investigated the breeding systems of the Ruschioideae taxa *Cheiridopsis initans*, *C. denticulata*, *Ruschia goodiae* and *Leipoldtia schultzei* and diagnosed obligate xenogamy in all of them, as almost no seeds developed following autogamous and geitonogamous pollinations. However, there are counterexamples questioning general self-incompatibility within the Aizoaceae: Partial to full self-fertility was observed in species of the genera *Diplosoma* (Hammer 1995), *Mitrophyllum* (Hammer 2004), *Carpobrotus* (Suehs et al. 2004) and, most importantly, also in *Delosperma* (Hammer and Liede 1990; Schröder 2008). After self-pollinations of *Lampranthus* the portion of pollen grains germinating on stigmas appeared to be lower than after cross-pollinations. However, pollen tubes grew towards the micropyles of ovules and in flowers of genotype II some pre-globular embryos were detected. The absence of seed formation indicates the occurrence of late-acting self-incompatibility (LSI), which is widespread among angiosperms and becomes manifest in fertilization failures or early embryo abort (Gibbs 2014).

Pollen tube guidance is not disturbed in intra- and intergeneric cross-combinations

Proven in many angiosperm species, stigma and pistil tissues function as effective barriers for in- and outbreeding by possessing various molecular recognition and rejection mechanisms (Swanson et al. 2004). Following cross-pollinations within the genera *Delosperma* and *Lampranthus* high numbers of pollen tubes grew towards the ovary and precisely targeted the micropyles of ovules (Fig. 2a, c). Surprisingly, also after reciprocal intergeneric combinations pollen tube inhibition or disorientation was not observed (Fig. 2b and d; Table 2). In recent years numerous details of the molecular interactions during the journey of pollen tubes to the ovules were clarified. In most studies *Arabidopsis*, *Brassica* ssp., *Zea mays* or *Torenia fournieri* (a plant with protruding embryo sacs) were utilized as model organisms (see Dresselhaus and Franklin-Tong 2013; Higashiyama and Takeuchi 2015 for reviews). Swanson et al. (2004) legitimately pointed out that, due to the enormous diversity of plant reproductive systems, details of pollination anatomy should be generally discussed species-specific. Nevertheless, the known key factors involved in pollen tube guidance are briefly outlined below. During different phases of preovular and ovular guidance, specific communication events between the pollen (tube) and tissues of the pollinated flower are required. The first phase includes pollen adhesion, hydration and germination. In plants with dry papillous stigmas such as *Arabidopsis* and the midday flowers investigated in the present study, pollen adhesion tends to be selective, facilitating early discrimination of incompatible mates (Swanson et al. 2004). For the hydration process pollen coat lipids as well as interactions between pollen (coat) and stigmatic proteins, e.g. phosphoinositides, were found to be essential (Dresselhaus and Franklin-Tong 2013). After germination, pollen tubes grow through the stigma and pass a transmitting tissue, which is leading to the ovary.

Experiments in *Arabidopsis* and *Torenia* have shown, that molecular crosstalk in the style does not only affect progress of pollen tube growth directly, but is also a prerequisite for subsequent guidance to micropyles. This concept was termed competency control (reviewed by Higashiyama and Takeuchi 2015). Aside from rather general signaling agents, such as Ca^{2+}, reactive oxygen species (ROS) and γ-aminobutyric acid (GABA), specific polymorphic-secreted peptides and small proteins (particularly cysteine-rich proteins, CRPs) were found to mediate the consecutive steps of pollen tube guidance (Dresselhaus and Franklin-Tong 2013). Emerging from the transmitting tract, competent pollen tubes receive directional cues, secreted by the synergids of their target ovules (Higashiyama and Takeuchi 2015). Up to that point the molecular interactions in cross pollinated flowers of *Delosperma* and *Lampranthus* appeared to be accomplished successfully. However, occasional observations of pollen tubes overgrowing in embryo sacs following *Lampranthus* x *Delosperma* combinations (Fig. 4b) indicate failures of pollen tube rupture and sperm delivery. This last prezygotic step is commonly known as pollen tube reception (see Kessler and Grossniklaus 2011 for a review). In some of the *Lampranthus* ovules, in which coiling pollen tubes were observed, synergid cells were still existent. Omission of synergid degeneration and, consequently, continuous secretion of attractant cues could explain why ovules were occasionally penetrated by multiple pollen tubes (Fig. 4a). The phenomenon of "polytubey" was primarily studied in *Arabidopsis*, in which it possesses an opportunity for the recovery of failed or incomplete double fertilizations (Beale et al. 2012; Kasahara et al. 2012) and is independently controlled by both, the egg cell and the central cell (Maruyama et al. 2013).

Early embryogenesis is aberrant in many intra- and intergeneric combinations

Ovule clearing followed by DIC microscopy proved to be a valuable tool to verify fertilization success or failure and to observe the progress of early embryogenesis in *Delosperma* and *Lampranthus*. In comparison to microtome sectioning, the method of ovule clearing is less laborious, but its applicability is unfortunately limited. Adjustments of ovule orientation on microscope slides were difficult and did not succeed in every case. As observations of events inside the embryo sacs were only possible in side view orientation, precise quantitative evaluations could not be performed. At the latest from 15 days after pollination, incubation in Hoyer´s solution did not suffice to bleach ovules completely, primarily because, as part of seed coat development, oxalate crystals were deposited in the integuments. In unpollinated flowers and unfertilized ovules, embryo sacs formed circular cavities in close vicinity to the micropyle, while those, in which zygotes or embryos were observed after cross-pollinations in most cases had distinctly enlarged longitudinally. In ovules penetrated by pollen tubes, but lacking embryonic structures, embryo sac expansion was not observed. This indicates that the process is induced by synergid degeneration and/or double fertilization. Following the cross-pollination *Delosperma* I x *Lampranthus* I embryo sacs had not enlarged in some ovules containing abnormal embryos (Fig. 4e). Thus, the lack of embryo sac expansion might be related to failures of central cell fertilization or endosperm development.

Only few published investigations on Aizoaceae embryogenesis are available. These comprise the taxa *Aptenia cordifolia*, *Delosperma cooperi* (Prakash 1967a), *Hereroa hesperantha* (Prakash 1967b), *Mollugo nudicaulis* (Ly-Thi-Ba et al. 1970) and *Trianthema* ssp. (Bhargava 1935; Cocucci 1961). In line with the observations made in the present study, early embryos of all investigated Aizoaceae genera corresponded to the Solanad type and suspensors were described as uniseriate or partly biseriate (e.g. Johri et al. 1992). Diverging numbers of suspensor cells were frequently observed in *Delosperma cooperi* (Prakash 1967a), so that suspensor morphogenesis might not be a significant indicator for normal or aberrant embryo development. The endosperm is of the nuclear type and cellularizes at the micropylar pole at the late globular stage (Prakash 1967a and b). In the present study endosperm nuclei were apparent in *Delosperma* ovules, but cellularization was not observed. In *Delosperma cooperi* the endosperm consists of only loosely arranged cells, which disappear almost completely during seed development (Prakash 1967a). With due regard to available information on midday flower embryogenesis, no deviations were detected after intrageneric *Delosperma* crossings. Observations of embryogenesis and seed development following *Lampranthus* cross-pollinations revealed that both processes required distinctly more time than in *Delosperma*. As none of the performed cross-combinations within *Lampranthus* turned out to be entirely compatible and comparable studies do not exist, it remains unclear whether or not the observed morphology of early *Lampranthus* embryos was representative for the genus.

Interestingly, noticeable differences of cell division patterns during suspensor and embryo development were detected following intergeneric crossings. Whether these deviant embryonic patterns could be indicative for inescapable seed abortion should be answered by analysis of later developmental stages using microtome sectioning. The genome sizes of the investigated *Delosperma* genotypes differed only marginally (1.18-1.27 pg/2C), whereas more significant differences were detected between *Lampranthus* genotypes (1.6-2.36 pg/2C; Braun und Winkelmann, submitted manuscript). After intergeneric fertilization events, the ratios of maternal/paternal genomes were highly imbalanced in some combinations, which is known to be a proximate cause of endosperm failures and seed abortion (Berger 1999). Commonly, hybrid dysfunctions and endosperm failures are ascribed to Bateson-Dobzhansky-Muller (BDM) incompatibilities, which rely on deleterious interactions of diverged parental transcripts (e.g. Rieseberg and Carney 1998; Lafon-Placette and Köhler 2015). Also epigenetic changes, which among other effects are related to transposable element (TE) activation and mobilization, are known to provoke postzygotic incompatibilities (Lohe and Chaudhury 2002; Michalak 2009). During the last decade, divergent or differentially expressed small RNAs attracted a relatively great deal of attention in this field of research and were repeatedly recognized as key factors in postzygotic alterations of gene expression as well as in epigenetic and transposable element regulation (reviewed by Ng et al. 2012).

Low hybrid vigor and hybrid breakdown indicate post-zygotic barriers

All seedlings which were derived from pollinations of *Lampranthus* I with *Delosperma* pollen lacked chlorophyll completely and discontinued growing before expansion of primary leaves. Various degrees of albinism were also observed in the offspring of intrageneric *Lampranthus* crossings. Those plants which continued growing and survived the transfer to greenhouse conditions constantly formed pale green leaves (Fig. 5; Table 4). In line with these observations, Hammer and Liede (1990) described absence of chlorophyll in seedlings derived from intrageneric cross-pollinations within *Psammophora* and *Conophytum*. Albinism is a frequently occurring and difficult to overcome postzygotic barrier in wide hybridizations (Kumari et al. 2009) and most likely the result of plastome-genome incompatibilities (PGI) (Greiner and Bock 2013).

Although the hybrid *Delosperma* IV x V (Fig. S1, Online Resource 1) would not have formed by conventional crossbreeding and sowing of seeds, further attempts to overcome postzygotic barriers *via* ovule culture were largely unsuccessful. Aside from chlorophyll deficiency, severe morphological abnormalities were observed among the obtained non-viable plantlets (Fig. S3, Online Resource 1). Future approaches should consider the optimal time point for ovule isolation during *in planta* maturation. Microtome sectioning of fertilized ovules could help to determine the duration after which organ differentiation is impaired. Furthermore, utilization of optimized nutrient media might be necessary. Inside the ovary loculi of midday flowers ovules are embedded in a viscous exudate, which in *Aptenia cordifolia* was found to be rich in proteins and polysaccharides, such as glucose, galactose and arabinose. During anthesis changes of the exudate composition, e.g. increase of protein concentrations, were detected (Kristen et al. 1980; Kristen and Liebezeit 1980). Presumably this secreted matrix plays key roles in ovular pollen tube guidance as well as during the first phases of seed development. Thus, implementation of nutrient media on the basis of ovary gland exudate composition could improve embryo rescue approaches in Aizoaceae species.

Rare hybridization success proven by molecular markers

The AFLP analyses confirmed hybridity for the tested progeny. Interestingly, at least two intergeneric hybrids resulted from the combination *Delosperma* III x *Lampranthus* I. These mainly corresponded to the maternal morphology and only few exclusively paternal banding patterns were detected by gel electrophoresis. Also the calculation of Jaccard similarity indexes revealed high genetic distance between the hybrids and their pollen parent (0.76-0.77). One of the intergeneric hybrids was analyzed flow cytometrically to determine its absolute DNA content (~ 1.2 pg/2C). Remarkably, the DNA content of the diploid pollen parent was previously estimated almost twice as high (2.36 pg/2C; Braun and Winkelmann, submitted manuscript). The small hybrid genome size and the low genetic contribution of the pollen parent could be explained by loss of paternal chromosomes after fertilization. Uniparental chromosome elimination is known for a long time to occur frequently in interspecific hybrids (e.g. Davies 1974). Genomic *in situ* hybridization (GISH) (Schwarzacher et al. 1989) could offer an opportunity to verify this assumption.

Although the success rate was very low, the analyses showed that wide hybridizations between midday flowers are possible in principle. Nevertheless, most hybrids obtained in this study showed low viability and many of them died early.

Conclusions

As pollen germination rates were high in the majority of tested parental genotypes, lacking pollen viability can be ruled out as potential cause for hybridization failures. Considering the huge amounts of pollen produced by each flower, even the relatively low germination rates in *Delosperma* genotype IV (9 %, Table 1) do not restrain its application in cross-pollinations. Generally, pollen management appeared a simple task in midday flower breeding, because the grains were relatively resilient and easy to harvest.

Genotypes of both genera were self-incompatible, but the underlying mechanisms were different. In *Delosperma* SI is most likely S-locus-controlled, because pollen tube growth already stopped on stigmas after self-pollinations. In contrast, *Lampranthus* genotypes showed late-acting self-incompatibility (LSI), in which error-prone fertilization or embryogenesis prevent the formation of seeds. In this genus SI might not be an absolute term, as the principles of LSI are only poorly understood (Gibbs 2014). For practical breeding, self-incompatibility can be advantageous, because it obviates the need for laborious flower emasculations.

Pollen germination and tube growth to the ovules were not inhibited, neither after intrageneric nor after intergeneric cross-pollinations. Thus, according to the current state of scientific knowledge, the molecular signals mediating pollen tube guidance to the micropyles appear congruent in all examined genotypes and have not diverged in the course of speciation. While most cross-combinations carried out within *Delosperma* were compatible, intrageneric pollinations in *Lampranthus* turned out to be difficult. As fertilizations and early embryogenesis were observed after all combinations of *Lampranthus* genotypes, postzygotic barriers such as endosperm failures or embryo abort are the most likely reasons for the lack of viable seeds. After intergeneric combinations, incompatibilities in molecular crosstalk became visible during pollen tube reception and double fertilization. Despite the fact that fertilizations failed in most ovules, intergeneric hybridizations repeatedly resulted in formation of embryos, which showed aberrant morphogenesis patterns. Albinism appeared to be a frequently occurring and late-acting postzygotic barrier in intra- and intergeneric hybridizations. To our knowledge, prospects to solve this problem do not exist.

Due to the fact that error-prone embryo morphogenesis was already observed before endosperm cellularization and taking account of the general low vigor of obtained hybrids, further embryo rescue approaches in the investigated group of plants are inauspicious. Taken together, the analyses of reproductive isolation mechanisms between Aizoaceae genotypes showed that the barriers can be overcome. Leaking reproductive barriers between Aizoaceae taxa might constitute one explanation for the fast diversification of the plant family and could also allow for the development of new midday flower cultivars.

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Figures

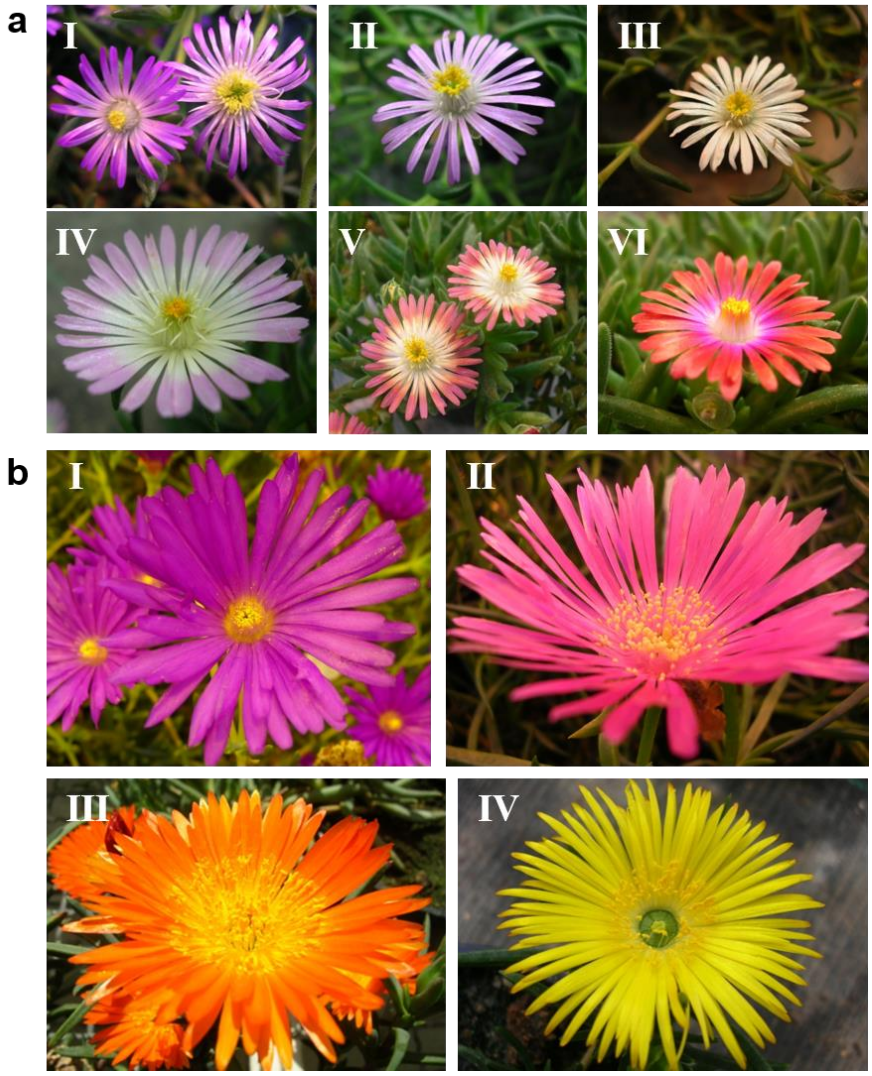

Fig. 1 Flowers of the investigated plants; **a** *Delosperma* genotypes I-VI; **b** *Lampranthus* genotypes I-IV; *Lampranthus* genotype V (not shown) exhibited no morphological differences to genotype II

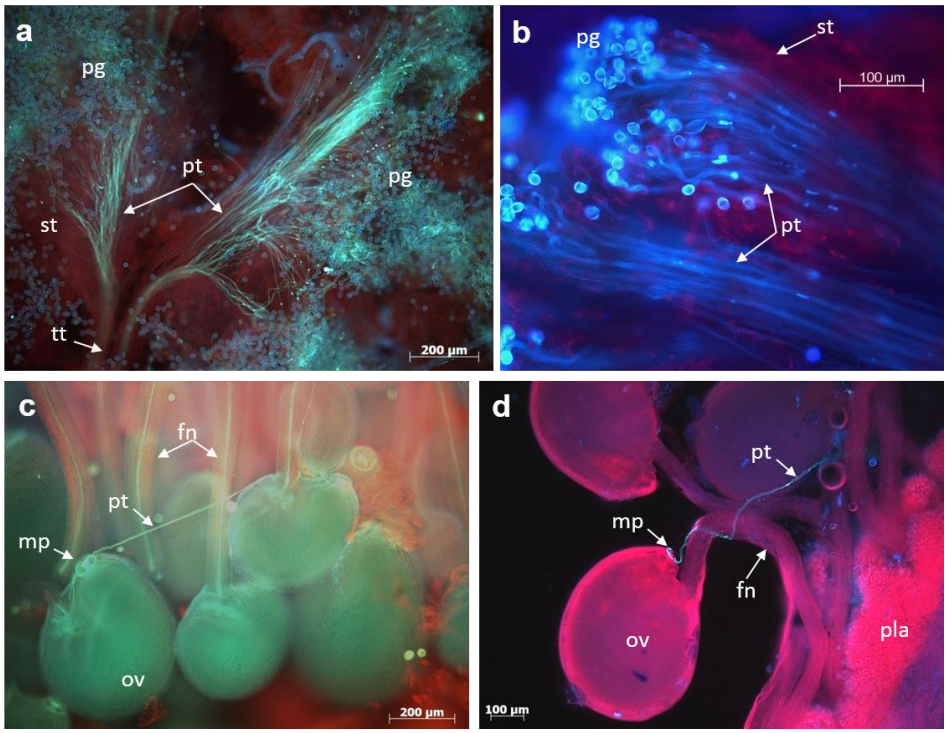

Fig. 2 Pollen tube growth on the stigma and in the transmitting tract 4 hours after cross-pollination of *Delosperma* II and III (**a**) and 24 hours after cross-pollination of *Lampranthus* I and *Delosperma* I (**b**); Pollen tubes entering the micropyles of ovules after the cross-pollination of *Lampranthus* IV x I (**c,** photo taken 5 DAP) and of *Delosperma* II and *Lampranthus* II (**d,** photo taken 5 DAP); Abbreviations: fn, funiculus; mp, micropyle; ov, ovule; pg, pollen grains; pla, placenta; pt, pollen tube; st, stigma; tt, transmitting tract

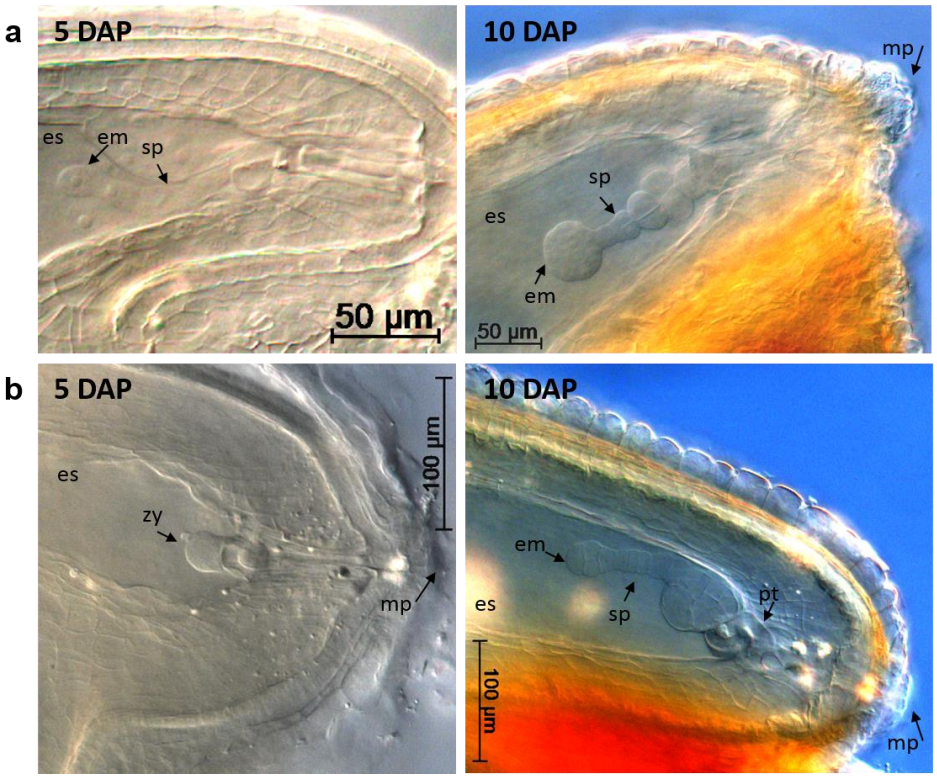

Fig. 3 Early embryogenesis in intrageneric cross combinations observed five and ten days after pollinations (DAP); **a** *Delosperma* V x VI; **b** *Lampranthus* I x II (left) and *Lampranthus* III x II (see text for details); Abbreviations: em, embryo; es, embryo sac; mp, micropyle; pt, pollen tube; sp, suspensor; zy, zygote

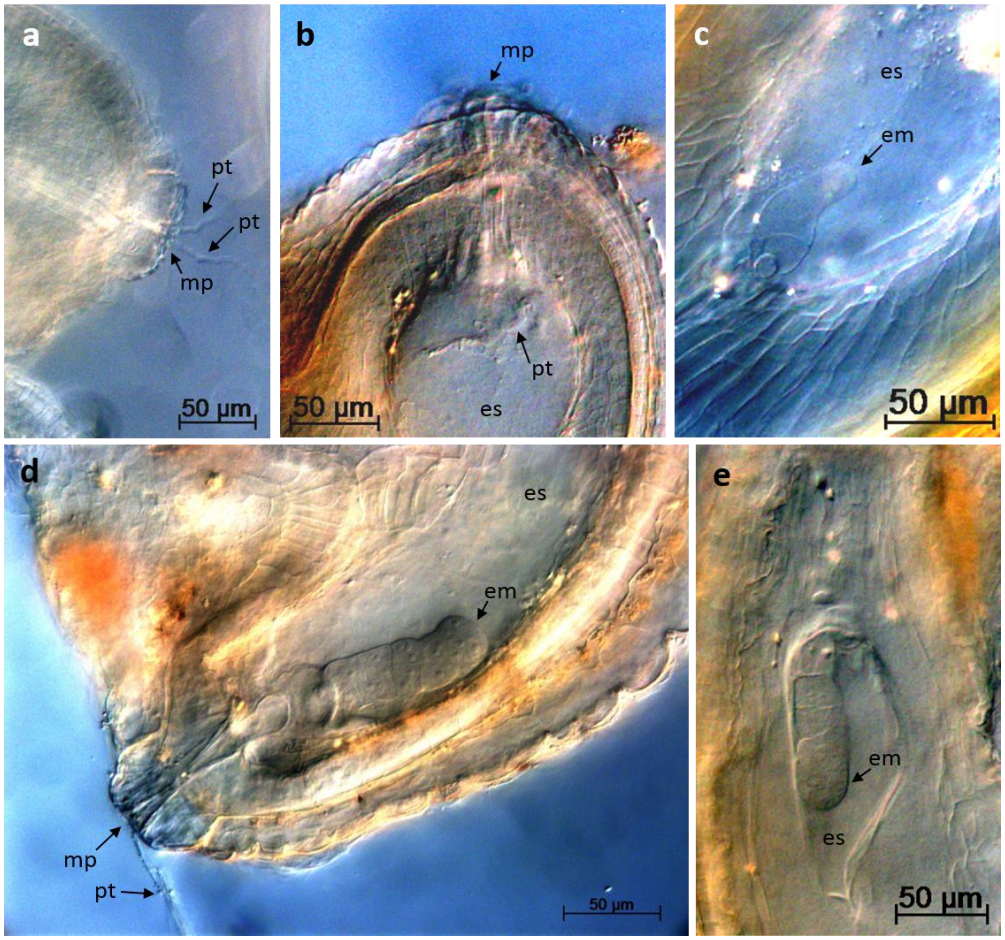

Fig. 4 Fertilization failures and abnormal early embryogenesis observed ten days after intergeneric cross-pollinations; **a-c** *Lampranthus* I x *Delosperma* I: **a** Micropyle penetrated by two pollen tubes ("polytubey"); **b** Pollen tube overgrowing in the embryo sac; **c** Underdeveloped proembryo; **d** *Delosperma* I x *Lampranthus* IV: Abnormally developed embryo; **e** *Delosperma* I x *Lampranthus* I: Abnormally developed embryo in a non-expanded embryo sac; Abbreviations: em, embryo; es, embryo sac; mp, micropyle; pt, pollen tube

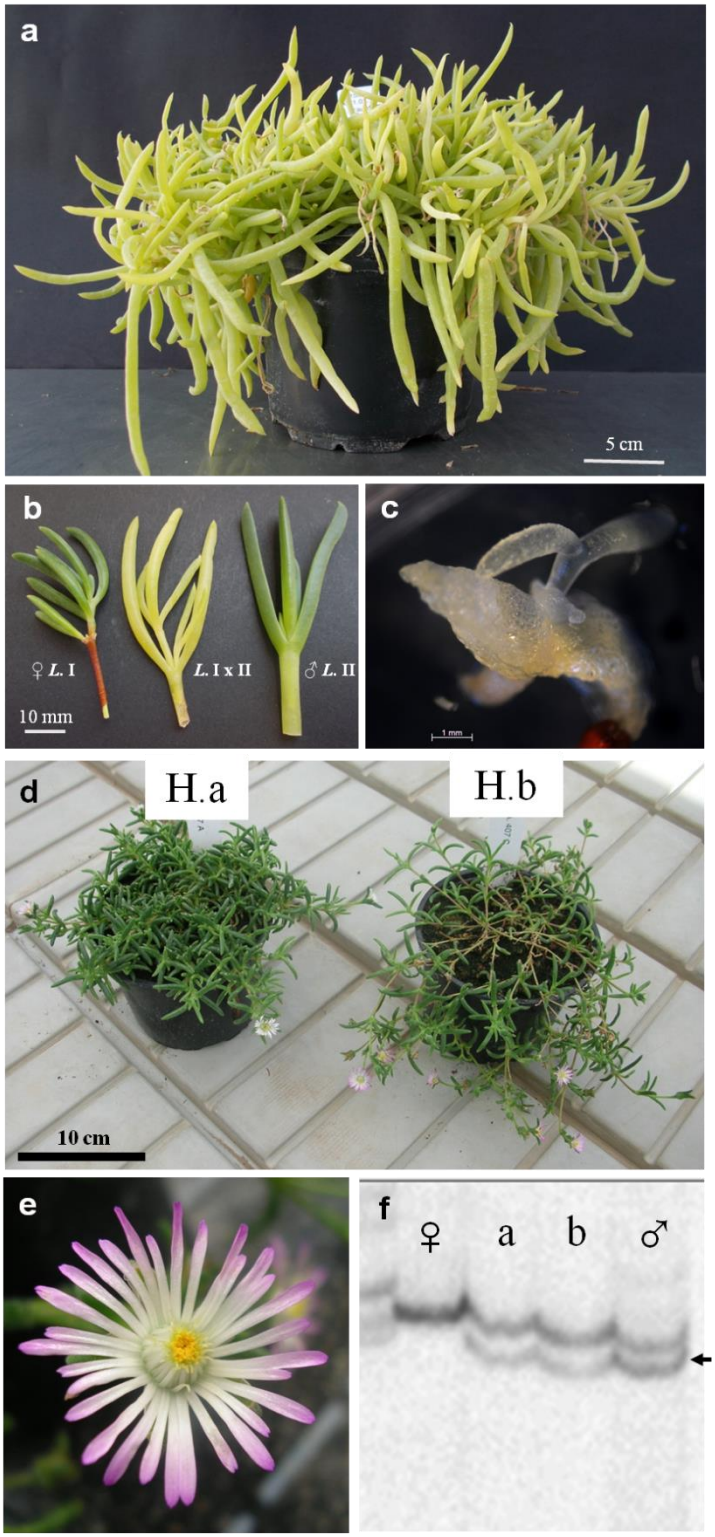

Fig. 5 Characteristics of hybrids originated from pollinations of *Lampranthus* genotype I (**a-c**) and from the intergeneric crossing *Delosperma* III x *Lampranthus* I (**d-f**); **a** Hybrid *Lampranthus* I x II (b) exhibiting atypical morphology and partial to complete leaf chlorosis (after one year of greenhouse cultivation); **b** Shoot tip comparison of the partially chlorotic hybrid *L.* I x II (e) and the non-chlorotic parental genotypes; **c** Completely chlorotic seedling originated from the cross-pollination *Lampranthus* I x *Delosperma* I; **d** Appearance of *Delosperma* III x *Lampranthus* I (H.a) and (H.b) one year after acclimatization to greenhouse conditions; **e** Flower of *D.* III x *L.* I (H.b) showing violet pigmentation, **f** Detail of AFLP banding patterns of parental and offspring genotypes, amplified with primer combination *Hind*III-ATT/*Mse*I-GGC; the arrow indicates DNA fragments, which are exclusively present in the hybrid and paternal band pattern

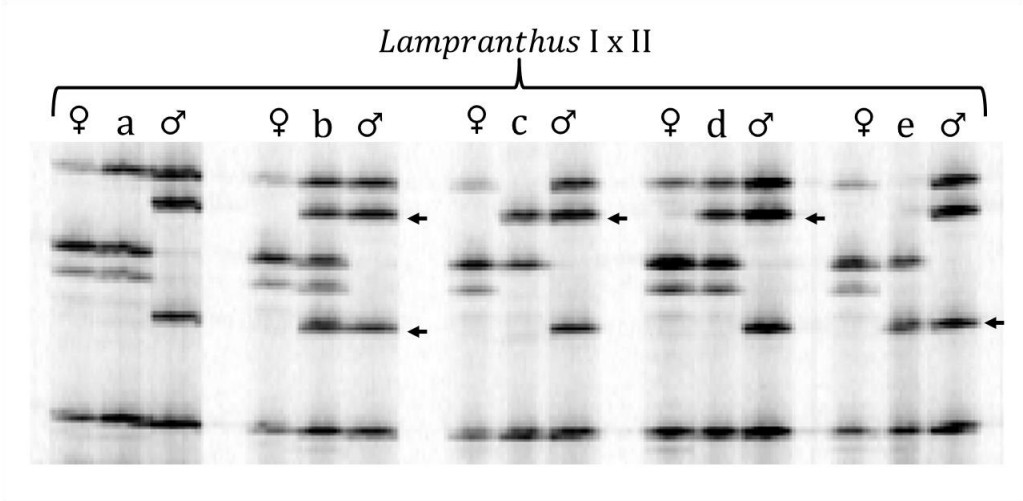

Fig. 6 Detail of AFLP banding patterns of *Lampranthus* I (♀), *Lampranthus* II (♂) and five hybrids (a - e), amplified with primer combination *Hind*III-CAT/*Mse*I-GAC; arrows indicate DNA fragments, which are exclusively present in the hybrid and paternal matrix

Tables

Table 1 *In vitro* germination (%) of fresh pollen *in vitro*; SD = Standard deviation of three technical replicates; * = tested without repetition

Genus/Hybrid	Genotype	Mean germination rate (%)	± SD
Delosperma	I	68	8.1
	II	68	7.3
	III	73	2.9
	IV	9	0.4
	V	73	5.4
	VI	58	2.3
Lampranthus	I	84	4.5
	II	58	5.4
	IV	87	3
Delosperma x *Delosperma*	IV x V	8	1.5
Delosperma x *Lampranthus*	III x I (b)	56*	-
Lampranthus x *Lampranthus*	I x II (a)	9	0.9
Lampranthus x *Lampranthus*	I x II (c)	8	2.8
Lampranthus x *Lampranthus*	I x II (d)	17	8.4

Table 2 Evaluation of pollen tube growth *in situ* following self-pollinations and intrageneric and intergeneric cross-pollinations by aniline blue staining; n = 5 flowers per combination and harvest date; displayed results corresponded to observations recorded 5 DAP

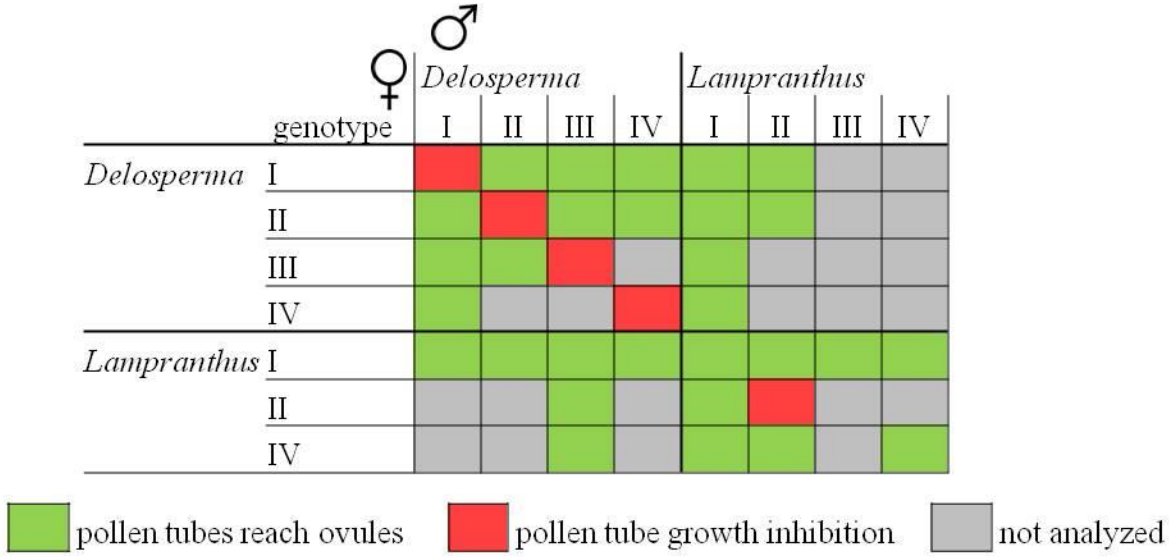

Table 3 Overview of performed cross-pollinations and results of embryogenesis observations by DIC microscopy; n = 5 ovaries per combination; observations recorded 10 DAP

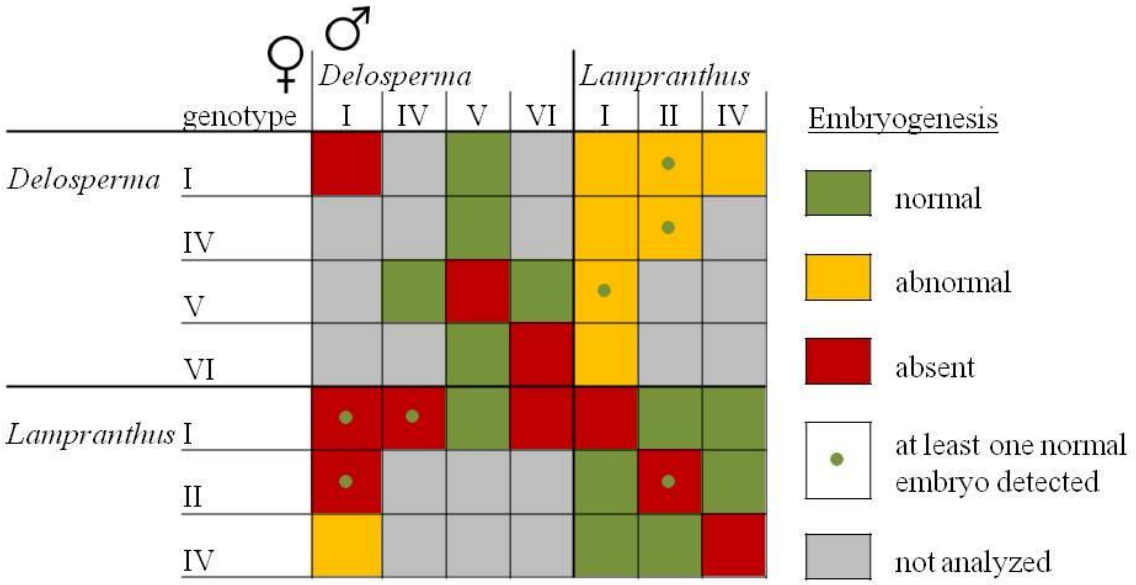

Table 4 Overview of capsule development, seed set, seed germination and leaf colour of seedlings following intra- and intergeneric cross pollinations; *SD* Standard deviation, *n.a.* not analyzed, *dg* dark green, *pg* pale green, *cc* completely chlorotic seedlings; coloration: yellow and blue, parental genotypes; orange and dark blue, intrageneric crossings; green, intergeneric crossings

Seed parent ♀	Pollen parent ♂	No. of pollinated flowers	No. of developed capsules (% of pollinated flowers)	Mean no. of seeds/capsule (SD)	seed germination (%)	leaf colour
Lampranthus I	*Lampranthus* I	10	0	0	n.a.	n.a.
	Lampranthus II	39	26 (67)	32 (22.7)	11	pg
	Lampranthus III	6	5 (83)	2 (1.8)	0	n.a.
	Lampranthus IV	26	24 (96)	11 (10.6)	15	pg
	Lampranthus V	19	18 (95)	24 (13.8)	53	pg
	Delosperma I	27	9 (33)	12 (9.8)	10	cc
	Delosperma II	39	12 (31)	14 (10.9)	5	cc
	Delosperma III	33	3 (9)	9 (2)	30	cc
	Delosperma IV	29	3 (10)	7 (11.3)	0	n.a.
	Delosperma V	10	4 (40)	5 (2.9)	20	cc
Lampranthus II	*Lampranthus* I	11	10 (91)	34 (27.6)	2	pg
	Lampranthus III	12	12 (100)	4 (5.1)	0	n.a.
	Lampranthus IV	7	6 (86)	1 (1.8)	0	n.a.
	Delosperma I	4	4 (100)	4 (4.7)	0	n.a.
Delosperma I	*Delosperma* I	3	0	0	n.a.	n.a.
	Delosperma II	27	20 (74)	132 (47.9)	45	dg
	Delosperma III	26	10 (38)	128 (41)	56	dg
	Delosperma IV	13	2 (15)	21	24	dg
	Lampranthus I	30	4 (13)	3 (4.4)	10	cc
Delosperma II	*Delosperma* I	28	15 (54)	62 (50.5)	45	dg
	Delosperma II	9	0	n.a.	n.a.	n.a.
	Delosperma III	5	5 (100)	102 (23.9)	42	dg
	Delosperma IV	3	3 (100)	24 (2.9)	11	dg
	Lampranthus I	15	0	n.a.	n.a.	n.a.
	Lampranthus II	2	0	n.a.	n.a.	n.a.
Delosperma III	*Delosperma* I	1	1 (100)	54	39	dg
	Delosperma II	23	19 (83)	150 (45.8)	31	dg
	Delosperma III	2	0	n.a.	n.a.	n.a.
	Delosperma IV	12	9 (75)	24 (10.2)	78	dg
	Lampranthus I	11	1 (9)	4	100	dg
Delosperma IV	*Delosperma* I	2	0	n.a.	n.a.	n.a.
	Delosperma III	10	0	n.a.	n.a.	n.a.
	Delosperma IV	3	0	n.a.	n.a.	n.a.
	Delosperma V	17	0	n.a.	n.a.	n.a.
	Lampranthus I	2	0	n.a.	n.a.	n.a.
Delosperma V	*Delosperma* V	2	0	n.a.	n.a.	n.a.
	Delosperma VI	5	5 (100)	59 (11.8)	94	dg
	Lampranthus I	4	0	n.a.	n.a.	n.a.
Delosperma VI	*Delosperma* V	5	4 (80)	45 (3.7)	89	dg
	Delosperma VI	2	0	n.a.	n.a.	n.a.
	Lampranthus I	1	0	n.a.	n.a.	n.a.

Table 5 Germination of ovules cultured following different durations of *in planta* maturation after cross-pollinations; *WAP* weeks after pollination; coloration: yellow and blue, parental genotypes; orange and dark blue, intrageneric crossings; green, intergeneric crossings

Seed parent ♀	Pollen parent ♂	Ovary harvest (WAP)	No. of cultured ovules	No. of germinations
Lampranthus I	*Lampranthus* II	3	327	1
		4	157	3
		5	236	4
	Lampranthus III	5	159	2
	Lampranthus IV	5	412	10
	Delosperma I	3	52	0
		4	208	1
		5	229	5
	Delosperma II	3	100	0
		4	80	0
		5	112	3
	Delosperma III	3	63	0
		4	37	0
		5	22	0
		6	62	1
	Delosperma VI	4	84	0
Lampranthus II	*Lampranthus* I	4	154	0
		5	137	73
		6	106	20
		7	91	8
	Lampranthus IV	4	94	0
		5	63	3
		6	34	0
		7	21	3
Delosperma IV	*Delosperma* II	3	25	11
	Delosperma V	3	42	12
		4	43	20

Table 6 Jaccard coefficients of genetic distances between the investigated genotypes obtained by AFLP analysis; 0 = no differences – 1 = no similarities; coloration of genetic relations: blue and yellow, parental genotypes; dark blue and orange, intragenric relations; green, intergeneric relations

		Parental genotypes						Hybrids									
		Delosperma			*Lampranthus*			*Delosperma*		*Delosperma* III x *Lampranthus* I		*Lampranthus*					
		III	IV	V	I	II	V	III x IV	IV x V	a	b	I x II a	I x II b	I x II c	I x II d	I x II e	I x V
Delosperma	III	0.00															
	IV	0.54	0.00														
	V	0.54	0.66	0.00													
Lampranthus	I	0.83	0.73	0.80	0.00												
	II	0.80	0.74	0.77	0.42	0.00											
	V	0.81	0.75	0.74	0.34	0.34	0.00										
Delosperma	III x IV	0.37	0.47	0.66	0.82	0.83	0.83	0.00									
	IV x IV	0.60	0.40	0.40	0.72	0.69	0.69	0.65	0.00								
Delosperma III x *Lampranthus* I	a	0.32	0.58	0.58	0.77	0.72	0.73	0.49	0.58	0.00							
	b	0.38	0.59	0.56	0.76	0.76	0.75	0.56	0.64	0.27	0.00						
Lampranthus	I x II a	0.82	0.74	0.76	0.23	0.23	0.31	0.82	0.72	0.79	0.80	0.00					
	I x II b	0.82	0.76	0.77	0.29	0.25	0.33	0.84	0.74	0.74	0.77	0.15	0.00				
	I x II c	0.82	0.74	0.78	0.27	0.26	0.32	0.85	0.73	0.76	0.76	0.14	0.18	0.00			
	I x II d	0.83	0.80	0.80	0.34	0.29	0.37	0.86	0.76	0.76	0.78	0.20	0.21	0.24	0.00		
	I x II e	0.83	0.75	0.78	0.28	0.13	0.18	0.84	0.72	0.77	0.79	0.10	0.16	0.15	0.11	0.00	
	I x V	0.80	0.79	0.72	0.31	0.23	0.32	0.86	0.73	0.74	0.71	0.23	0.25	0.20	0.32	0.20	0.00

Online Resource 1 (Supplemental Material)

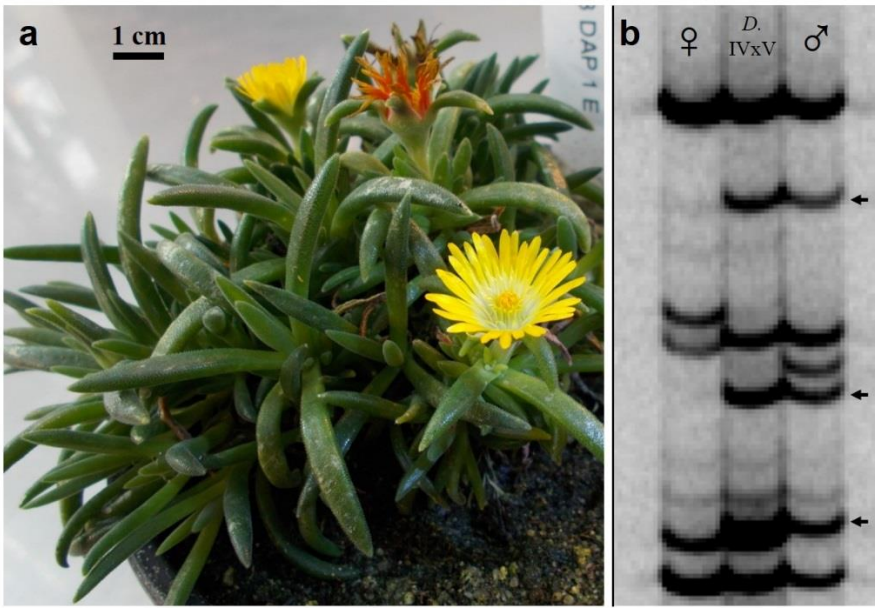

Fig. S1 **a** *Delosperma* IV x V hybrid generated via ovule culture (4 WAP); photo taken eight months after acclimatization to greenhouse conditions; *WAP* weeks after pollination (= duration of *in planta* maturation); **b** Detail of AFLP banding patterns of *Delosperma* IV (♀), V (♂) and the obtained hybrid, amplified with primer combination *Hind*III-CAT/*Mse*I-GAC; the arrows indicate DNA fragments, which are exclusively present in the hybrid and paternal banding pattern

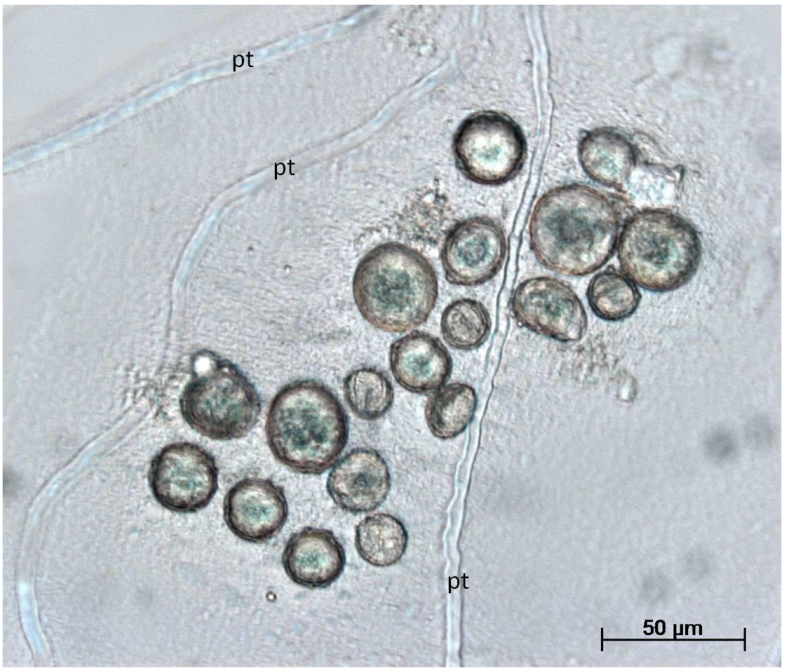

Fig. S2 Variable pollen grain diameters of the hybrid *Lampranthus* I x II (c) observed during the evaluation of *in vitro* germination tests; pt, pollen tube

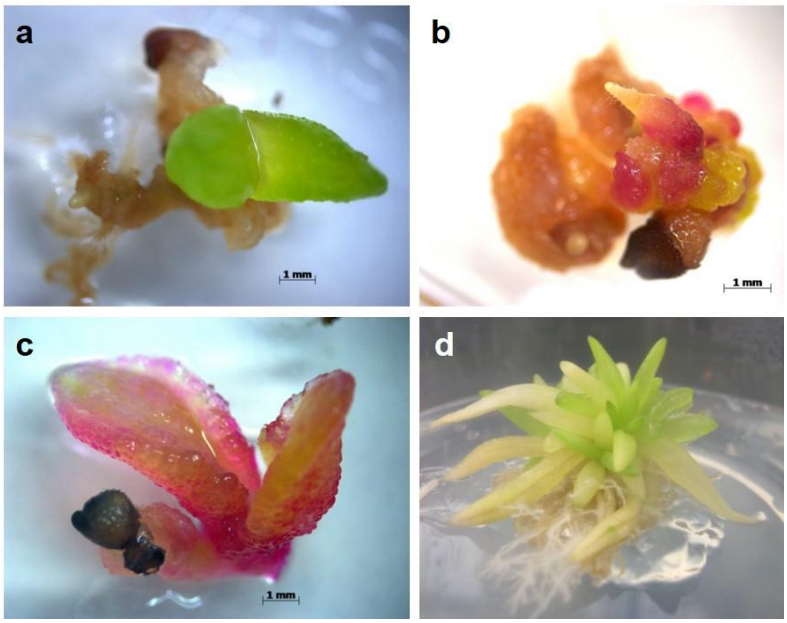

Fig. S3 Abnormal plantlets emerged from cultured ovules; **a** *Lampranthus* I x II (5 WAP); **b** *Lampranthus* I x IV (5 WAP); **c** *Lampranthus* I x *Delosperma* III (6 WAP); **d** *Delosperma* IV x V (4 WAP); *WAP* weeks after pollination (= duration of *in planta* maturation)

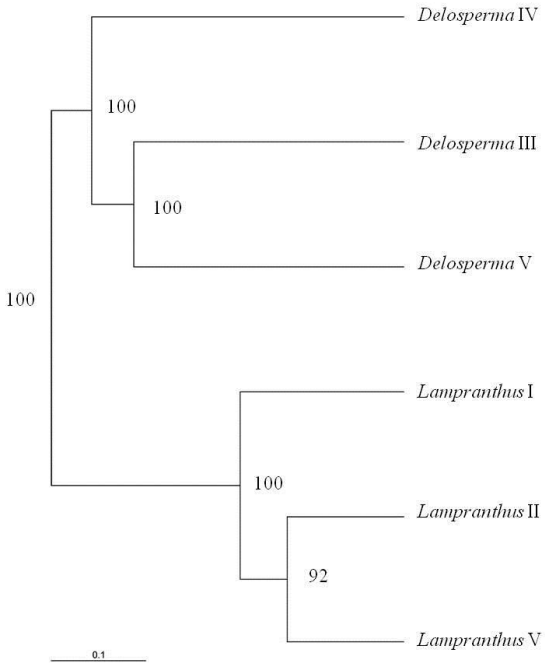

Fig. S4 Genetic relationships of six parental genotypes obtained by AFLP analysis and calculation of Jaccard similarity indexes; bootstrap values are given next to the branches of the Neighbor-joining phenogram

Chapter 4: Manuscript III

Cytological Investigations in Midday Flowers (Aizoaceae) Reveal High DNA Contents in Different Somatic Tissues and Potential Occurrence of Unreduced Male Gametes

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Cytological Investigations in Midday Flowers (Aizoaceae) Reveal High DNA Contents in Different Somatic Tissues and Potential Occurrence of Unreduced Male Gametes

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Keywords: *Delosperma*, *Lampranthus*, endoreduplication, endocycling, flow cytometry, polyploidization

Abstract

Breeding, possibly including polyploidization, in the midday flowers (*Aizoaceae*) genera *Lampranthus* and *Delosperma* is of interest for developing novel drought tolerant bedding plants. However, cytological knowledge on these plants is scarce. Flow cytometric investigations on different genotypes of *Lampranthus* and *Delosperma* revealed that most organs of these plants predominantly consist of endoreduplicated cells with up to five different DNA contents (2C-32C). Endoreduplication (endocycling) is a process involving one or several rounds of nuclear DNA synthesis without chromosomal and cellular division. The highest proportions of endoreduplicated cells were detected in petaloid staminodes and mature succulent leaves (up to 95 %), whereas only relatively low percentages were found in young leaves and roots (≈ 23-56 %). A comparison of greenhouse and *in vitro* grown plant organs in respect of their endoreduplication patterns only showed marginal differences. How endocycling affects *in vitro* shoot regeneration and/or chromosome doubling through antimitotic agents remains to be clarified. Further observations on midday flowers of these genera revealed that pollen grains of identical genotypes had various diameters, possibly indicating spontaneous production of unreduced male gametes (2n pollen). In addition microscopic analyses showed that exceptionally large pollen grains also contained larger sperm nuclei. Flow cytometric measurements of pollen nuclei isolated from germinated tricellular grains resulted in the detection of 1C (haploid), 2C and even low percentages of 3C particles. Ongoing microscopic analyses of microsporogenesis, microgametogenesis and the appearance of gametes in pollen tubes should clarify the question whether the detected 2C and 3C signals were caused by unreduced male gametes or physically connected pollen nuclei (male germ units).

INTRODUCTION

The Aizoaceae is the largest of all succulent plant families. Its subfamily Ruschioideae alone, which also includes the genera investigated in this study, covers almost 1600 exclusively perennial species, most of them endemic to southern Africa (Klak et al. 2003). Their drought tolerance, their diversity of aridity adapted growth forms and finally their intensively colored flowers make many Aizoaceae species attractive candidates as ornamental plants. Although some non protected varieties are offered in garden centers, selective breeding of these plants is still in its infancy.

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Aims of this study were (1) to characterize the occurrence of endoreduplication in different plant organs and (2) to ascertain whether the investigated genotypes produce unreduced gametes spontaneously.

MATERIALS AND METHODS

Plant material and cultivation

Three genotypes, which should also be used in future crossing experiments, were included in this study: *Delosperma cooperi* and two genotypes out of the genus *Lampranthus* whose exact phylogenetic background and breeding history were unknown. With regard to their growth forms, leaf shapes and flower morphologies, and in comparison with species descriptions by Van Jaarsveld and De Pienaar (2004), *Lampranthus* genotype 1 showed striking similarities to *L. aureus*, and *Lampranthus* genotype 2 to *L. roseus* (Fig. 1). Cuttings were rooted and cultivated in peat/sand (3:1) substrate. Mean daily temperature was adjusted to 20 °C by automatic ventilation and shading. Relative humidity ranged between 40 and 60 %. Throughout the year daily photoperiods were extended to a minimum of 16 hours by the use of high pressure sodium lamps (approx. 1 lamp/4m^2; Philips MASTER Agro 400W) which were also used for supplementary lighting when solar irradiation fell below 25 klx. Plants were irrigated manually and fertilized once a week with 0.1 % Hakaphos rot © (Compo, 1N:1.5P:3K). For the comparison of endoreduplication patterns in greenhouse and *in vitro* grown plant organs, surface sterilized cuttings of *Delosperma cooperi* were rooted and cultivated in MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose, which was solidified with 3 g/l Gelrite® (Duchefa, Haarlem, Netherlands). As Gelrite® raised the pH value of the medium, it was adjusted to 5.8 using 0.1 M HCl. Planted vessels were placed at 23 °C and a photosynthetic photon flux density of approximately 40 µmol m^{-2} s-^{1} for five weeks before the flow cytometric analyses.

Determination of organ-specific endoreduplication patterns

Each plant organ was investigated in at least five repetitions. Small amounts of tissue were chopped with razor blades in 400 µl of a nuclei extraction buffer (PreciseP Partec, Münster, Germany). After two minutes incubation the suspensions were passed through 30 µm Celltric filters (Partec). Subsequently 1 ml of DAPI buffer (PreciseP Staining Buffer, Partec) was added to the nuclei suspensions which were analyzed with a flow cytometer (CyFlow Ploidy Analyzer, Partec). Signal amplifications (gain) were chosen individually to make sure that all nuclei sizes were displayed by the software. Recorded nuclei numbers ranged between 2000 and 15000 in one measurement, depending on the type of tissue. Based on the total numbers of nuclei recorded in one measurement, the percentages of the different nuclei types in the particular organ were calculated.

Pollen size measurements and determination of pollen type

Lampranthus genotype 2 was chosen for pollen analyses, because it produced sufficient amounts of flowers over a longer period of time. Pollen grains were dispersed on solidified germination medium (Brewbaker and Kwack 1963) in Petri dishes using a brush. After a hydration time of 30 minutes the grain diameters were measured under a microscope (AxioScope A1, Zeiss, Jena, Germany) using the Zeiss AxioVision software. *Lampranthus* genotype 2 pollen grains were measured in three repetitions (each comprised at least 300 pollen grains).

Fresh pollen grains of midday flowers have a thick exine and are covered with a layer of sticky exudate hindering microscopic insights. Grains of all genotypes were incubated in 100 µl nuclei extraction buffer for a few hours to dissolve it. Afterwards 200 µl

of DAPI staining buffer (Partec) were added and the nuclei were observed by fluorescence microscopy (358 nm, Zeiss Filterset 02).

Flow cytometric analyses of pollen nuclei

At three different dates, pollen of 15 completely opened flowers was collected in Petri dishes and dried for 24 h at room temperature. Using a brush the pollen was dispersed on solidified germination medium (Brewbaker and Kwack 1963) and incubated at room temperature for 24 h. In a next step the layer of pollen grains and tubes was carefully scratched from the surface of the germination medium using a razor blade and transferred into 400 µl of the Partec nuclei extraction buffer. Afterwards grains and tubes were thoroughly chopped with razor blades to extract the containing nuclei. Nuclei suspensions were passed through 10 µm Celltric filters (Partec) to remove ungerminated pollen grains and debris. Afterwards, 800 µl of DAPI buffer (PreciseP Staining Buffer, Partec) were added to each sample tube. Each measurement comprised at least 6500 recorded particles. To ensure that nuclei with different C-values appear at their appropriate positions in the histograms, additionally somatic reference material (young leaves) was measured with the same signal amplification.

RESULTS

Organ-specific endoreduplication patterns

Organ-specific flow cytometric analyses of *Lampranthus* genotype 1 revealed that its tissues consisted of cells with up to five different DNA contents (2C-32C, Fig. 2). Because the method did not allow a discrimination between nuclei in the G_2 phase of a normal mitotic cell cycle and endocycling cells with a 4C DNA value, all recorded 4C nuclei were regarded as endoreduplicated below. The lowest percentage of endoreduplicated cells was detected in roots (≈ 23 %, 4C-8C), the highest in petaloid staminodes (≈ 95 %, 4C-32C). Very young leaves (max. 5 mm long) consisted of approximately 51 % endoreduplicated cells (4C-16C) whereas this proportion rose to approximately 87 % (4C-32C) in fully developed leaves. Similar nuclei proportions were ascertained in two further *Lampranthus* genotypes (data not shown).

Only four nuclei classes were detected in the investigated organs of *Delosperma cooperi* (Fig. 3). In greenhouse grown young leaves of *D. cooperi* the mean percentage of endoreduplicated cells was 17 % (4C-8C). In mature leaves approximately 63 % of the cells had a C-value higher than 2 (4C-16C). In roots approximately 34 % of the cells were found to be endoreduplicated (4C-16C). Analyses of *in vitro* grown plant organs led to almost identical results (Fig. 3).

Pollen analyses

Almost normally distributed, the determined pollen diameters of *Lampranthus* genotype 2 ranged between 26 and 45 µm, rendering a classification of ploidy levels according to pollen size impossible (Fig. 4). Fluorescence microscopic observations revealed that pollen of all investigated genotypes was shed in a trinuclear state. The flow cytometric analyses of isolated *Lampranthus* genotype 2 pollen nuclei led to the detection of particles corresponding to 1C, 2C and 3C nuclei at each repetition (Tab. 1). The proportions of haploid (1C) nuclei ranged between 85.6 and 91.1 % and those of 2C particles between 7.5 and 10.8 %. The highest detected proportion of 3C particles was 4.9 %.

DISCUSSION

The cell cycle modification leading to polysomaty in angiosperms most frequently is referred to as endoreduplication (also endoreplication or endocycling). It involves one or several rounds of nuclear DNA synthesis without chromosomal and cellular division

(D`Amato 1984, Breuer et al. 2014). Polyploidization in its narrow sense implies an amplification of chromosome numbers, but during endocycling doubled sister chromatids remain bundled in a constant number of polytene chromosomes (Šesek et al. 2005, Bauer and Birchler 2006, Bourdon et al. 2011, also see the recent review of Breuer et al. 2014). For this reason the common, but misleading term "endopolyploidy" is not used below. Another cytological process indeed generates increased chromosome numbers in polysomatic tissues: Endomitosis, which has been only rarely detected in plants, involves retention of the nuclear membrane and omission of cytokinesis (D`Amato 1984).

Wulff (1940) conducted microscopic analyses on roots of various Aizoaceae species and reported for the first time on diverging cell nuclei sizes in this family. De Rocher et al. (1990) analyzed the annual midday flower *Mesembryanthemum crystallinum* flow cytometrically and in this way verified up to six different cell DNA contents in some of its organs. As the DNA content of somatic *M. crystallinum* cells was estimated to be lower than 1 pg/2C, they concluded that developmentally regulated polysomaty could be a common feature of CAM plants with small genomes. With regard to genome size Soltis et al. (2003) classified the entire family Aizoaceae as `very small´ (< 1.4 pg/1C).

In the present study four different cell nuclei classes were detected in *D. cooperi* and five in a *Lampranthus* genotype (2C-32C). According to this, the extent of endoreduplication in these plants was slightly lower than in *M. crystallinum*. In mature leaves, flower organs, roots and cotyledons of this annual midday flower De Rocher et al. (1990) even detected small proportions of cells with a DNA content corresponding to 64C. Presumably endoreduplication plays a key role in the formation of succulent leaf tissues and therefore contributes to the drought tolerance of midday flowers. In contrast to the roots of *M. crystallinum*, in which De Rocher et al (1990) detected a high degree of polysomaty, the roots of the plants investigated in this study only contained low proportions of endoreduplicated cells. The remarkable extent of endoreduplication in petaloid staminodes probably enables the investigated genotypes to form large pollinator attracting organs.

Plant parts of *D. cooperi* grown under *in vitro* conditions showed endoreduplication patterns which were almost identical to those detected in greenhouse grown plants. Presumably endoreduplication is under intrinsic control and not or only marginally affected by environmental conditions. Palomino et al. (1999) investigated the influence of long-term *in vitro* culture (with and without auxins) on the genome stability and endoreduplication patterns of the Cactaceae species *Mammillaria san-angelensis*. Even after seven years of *in vitro* culture neither somaclonal variations nor endoreduplication patterns different from the wild-type (2C-16C) were identified.

How endocycling affects *in vitro* shoot regeneration and/or chromosome doubling through antimitotic agents is of particular interest for breeding approaches in midday flowers. For shoot regeneration mitotically active cells in the explants are a basic requirement. In contrast to endomitotic cells, endocycling cells containing polytene chromosomes were thought to lose their ability to divide (D`Amato 1964, Nagl 1981). Palomino et al. (1999) used polysomatic tissue of *Mammillaria san-angelensis* as explant for *in vitro* shoot regeneration. They detected that all regenerated shoots contained 2C cells and concluded that 2C cells existing in the explant served as starting point for organogenesis.

The formation of unreduced male gametes (gametes with a somatic chromosome number or higher, also referred as 2n gametes) in most described instances relied on meiotic aberrations. Although the specific cytological processes are diverse, the non-reduction mostly results from omission of the first (first division restitution, FDR) or second (second division restitution, SDR) meiotic division (see reviews of Bretagnolle

and Thompson 1995, Younis et al. 2014). Microscopic analyses revealed that *Lampranthus* as well as *Delosperma* genotypes shed pollen grains with considerably varying diameters. In some cases also nuclei with visibly larger diameters were observed in exceptionally large pollen grains (diameter > 35µm in case of *Lampranthus* genotype 2) after DAPI staining. Because a positive correlation between DNA content and cell volume is normally also assumed for pollen grains (Bretagnolle and Thompson 1995), the various pollen diameters in midday flowers could indicate the spontaneous production of unreduced male gametes. A morphological screening to detect the frequency of 2n pollen, as performed in many other studies (Bretagnolle and Thompson 1995), was not successful in the investigated *Lampranthus* genotype, because the measured diameters could not be classified into two or three size ranges.

The measurements of pollen nuclei isolated from pollen tubes of a *Lampranthus* genotype led to the formation of three peaks corresponding to DNA contents of 1C, 2C and 3C. The 1C peaks definitely resulted from haploid sperm nuclei and/or vegetative nuclei. The relatively high proportions of detected 2C particles allow more than one interpretation: (1) As initially assumed, these particles might have been unreduced pollen nuclei. This should be confirmed in future crossing experiments or meiotic studies. (2) Theoretically, the 2C peaks could have been formed by generative pollen nuclei in the G_2 phase of the cell cycle. Though, in numerous microscopic observations binuclear pollen grains were never detected after flower opening. Finally, the detection of small proportions of particles corresponding to a 3C DNA content in *Lampranthus* samples allows a third interpretation. Most likely the 3C peaks resulted from physically associated pollen nuclei, so-called male germ units (MGUs) (for a review see McCue et al. 2011). Hirano and Hoshino (2009) investigated male gametogenesis in *Alstroemeria aurea*. They demonstrated that the connection between both sperm cells and/or the connection between one sperm cell and the vegetative nucleus were partly consistent in an extraction buffer. Furthermore, flow cytometric analyses of the MGUs resulted in peaks at the 3C position. (3) Thus, partly consistent MGUs (e.g. paired sperm cells) could be a further explanation for the detection of 2C particles in analyses of *Lampranthus* pollen. Projected microscopic analyses should clarify, if pollen nuclei of midday flowers remain associated in pollen tubes and nuclei isolates.

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Tables

Table 1. Flow cytometrically determined proportions (%) of different DNA contents (C-levels) in *Lampranthus* genotype 2 pollen nuclei; pollen samples were taken at three different dates; each sample consisted of pollen extracted from 15 flowers; SD = Standard deviation of seven measurements (%).

	18.02.2014		25.02.2014		19.03.2014	
	%	SD	%	SD	%	SD
1C	85,6	2,6	85,7	3,1	91,1	2,6
2C	10,8	1,3	9,4	1,5	7,5	2,0
3C	3,6	1,8	4,9	1,7	1,4	0,7

Figures

Fig. 1. Flowers of the investigated plants; *Delosperma cooperi* (A), *Lampranthus* genotypes 1 and 2 (B and C).

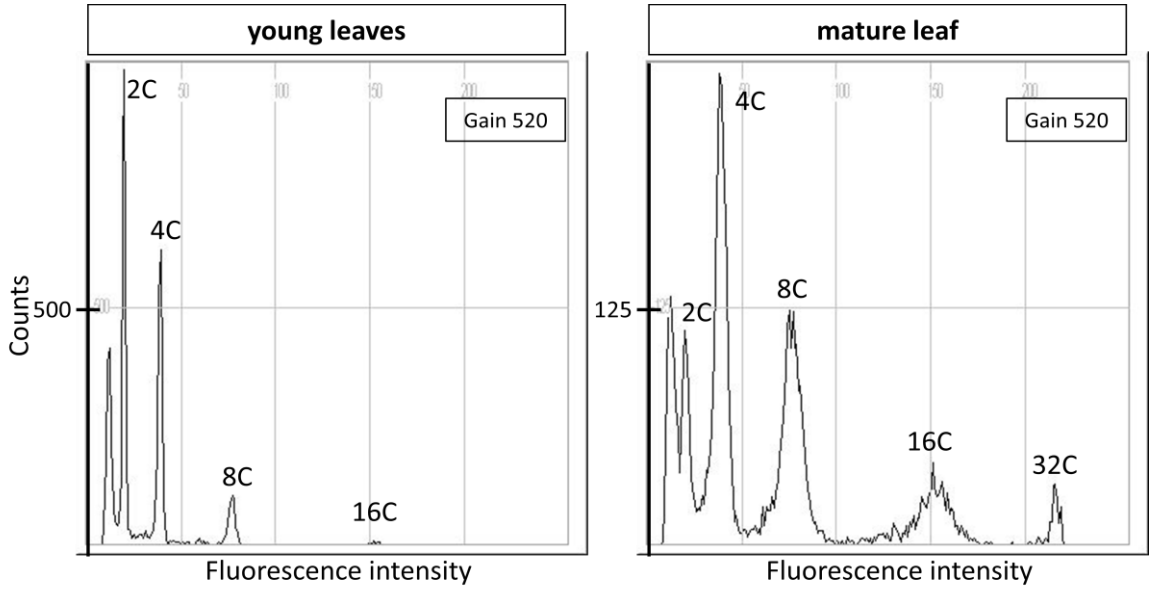

Fig. 2. Flow cytometric histograms resulting from analyses of young and mature leaves of *Lampranthus* genotype 1; peaks at the very left were caused by cell debris and/or extranuclear DNA.

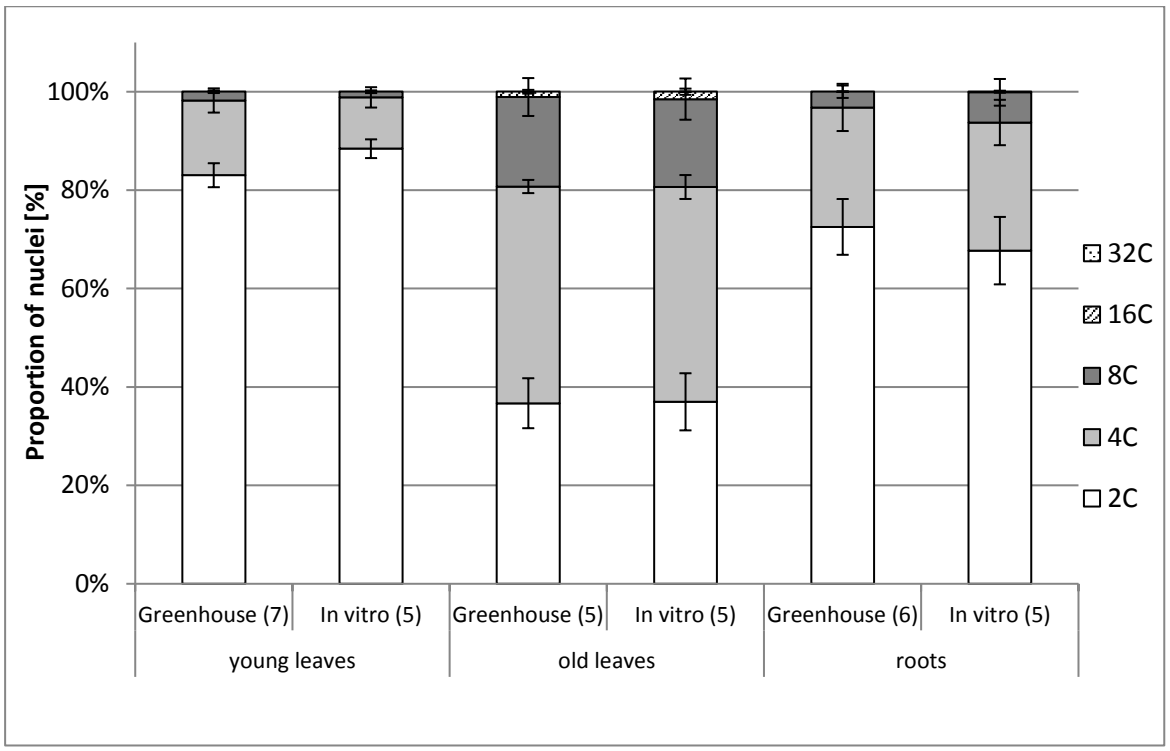

Fig. 3. Proportion of cell nuclei with different ploidy levels in various tissues of *Delosperma cooperi*; Comparison of greenhouse and in vitro grown material; numbers of analyzed samples in brackets, I = SD.

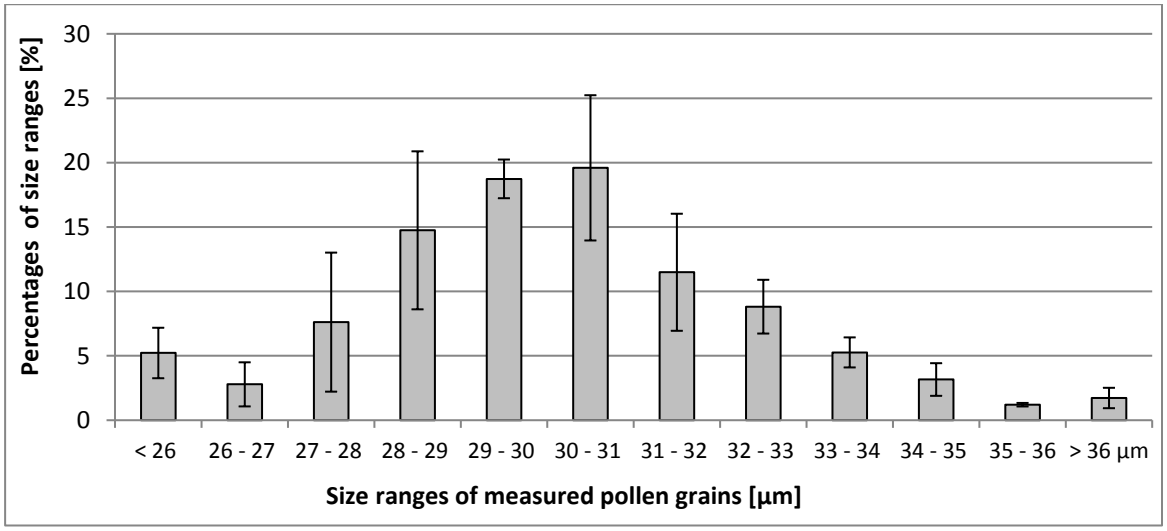

Fig. 4. Size ranges of measured pollen grains (*Lampranthus* genotype 2) and their percentages of the pollen population; mean values were calculated from results of three repetitions (each comprised at least 300 measured grains; I = SD); pollen grains < 26 µm were expected not to be hydrated completely; the largest determined diameter was 45 µm.

Chapter 5: Manuscript IV

Flow cytometric analyses of somatic and pollen nuclei in midday flowers (Aizoaceae)

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Abstract - Endoreduplication (continuous DNA replication without chromosomal or cellular division) is known to be extraordinarily pronounced in a number of succulent plants, including midday flower species. Cytological investigations in different genotypes of the Aizoaceae genera *Lampranthus* and *Delosperma* were performed with three objectives: Characterization of endoreduplication in several plant organs, determination of absolute DNA contents (pg/2C) and screening for unreduced male gametes. Flow cytometric determinations of absolute DNA contents using internal standards were carried out on nuclei extracted from pollen tubes. Further analyses of pollen nuclei were intended to verify the occurrence of unreduced gametes in the two genera. In contrast to the majority of angiosperms, in which endoreduplication is often restricted to certain tissues or cell types, the investigated succulents primarily consisted of cells with elevated DNA contents. Absolute DNA contents ranged from 1.6-2.36 pg/2C in *Lampranthus* and from 1.18-3.68 pg/2C in *Delosperma*. In flow cytometric analyses of pollen nuclei, signals corresponding to 1C, 2C and 3C DNA levels were detected. Microscopic observations of sample suspensions revealed that, instead of unreduced gametes, pairs of sperm nuclei and intact male germ units (MGUs) were the primary cause for the detection of signals higher than 1C. Thus, flow cytometric pollen nuclei analysis turned out to be a valuable tool to determine cell DNA contents in endocycling plants, but entails the risk of false-positive results in screenings for unreduced gametes due to persistent nuclei associations.

Key words: *Delosperma*; DNA content; endoreduplication; *Lampranthus*; male germ unit; unreduced gametes

Introduction

The Greater Cape Floristic Region (GCFR) in South Africa and Namibia is the main habitat of a remarkable succulent plant family, the Aizoaceae, often better known as midday flowers. The midday flower genera *Lampranthus* and *Delosperma* are two of about 100 taxa within the large subfamily Ruschioideae. Both belong to the Ruschieae tribe, which covers approximately 1600 species of succulent perennials (Klak et al. 2013) and, interestingly, was identified as the most rapidly diversifying known group of plants (Klak et al. 2004). The genus *Lampranthus* consists of erect to creeping shrubs and primarily occurs in winter rainfall areas near the Cape of Good Hope. In contrast, the genus *Delosperma* is more widely distributed within southern Africa and mostly occurs in summer rainfall regions. Among *Delosperma* species shrubby as well as mat-forming growth forms can be found (Hartmann 2001).

Aside from a botanical point of view, members of both genera are also attractive as ornamental plants, particularly because of their intensively coloured flowers and their tolerance to drought. However, so far only few efforts were made in directed breeding and development of new midday flower cultivars. One reason might have been that cytological knowledge on these plants that could facilitate the prediction and overcoming of hybridization barriers is scarce. For example, Klak et al. (2013) pointed out that relatively few chromosome counts have been published for the Aizoaceae. Genome size data, to our knowledge, have so far only been available for the species *Mesembryanthemum crystallinum* (De Rocher et al. 1990; Bennett and Leitch 1995), which belongs to a different subfamily (Mesembryanthemoideae) than the genera investigated in this study. De Rocher et al. (1990)

attracted a relatively great deal of attention by flow cytometric data obtained for *M. crystallinum* and succulents from other families. Their analyses revealed that polysomaty, i.e. the occurrence of different cell DNA contents within one and the same organism, is exceptionally pronounced in CAM plants (carbon fixation through Crassulacean Acid Metabolism) with small genome sizes. The cytological process behind polysomaty in angiosperms is referred to as endoreduplication or endocycling. In this mode of cell cycle, nuclear DNA amounts are doubled repeatedly in the absence of cytokinesis (D´Amato 1984; Breuer et al. 2014). Endoreduplication leads to polyteny, i.e. multiplied sister chromatids in a constant number of chromosomes (Šesek et al. 2005; Bauer and Birchler 2006; Bourdon et al. 2011), and may not be confused with polyploidization in its narrow sense (defined as the possession of three or more complete sets of chromosomes; Ramsey and Schemske 1998). However, it utilizes molecular regulators, which also hold key positions in mitosis, including cyclins (CYC), cyclin-dependent kinases (CDK) and CDK inhibitors. During endocycles these regulators are modified transcriptionally and/or post-translationally (Breuer et al. 2014). Besides other effects, endoreduplication results in higher cell volumes and faster cell expansion (e.g. Jovtchev et al. 2006; Hayashi et al. 2013). For succulents, this was postulated as a strategy for adaption to harsh environments (De Rocher et al. 1990; Del Angel et al. 2006). Within species boundaries, also the occurrence of narrow sense polyploids has been described in the Aizoaceae (e.g. Albers and Haas 1978; Hartmann 1986). In practical plant breeding, *in vitro* polyploidization by the use of spindle inhibitors is a widely applied technique. However, only little is known about the performance of endoreduplicative tissues in such approaches, which include shoot regeneration from single cells. Generally, cells containing polytene chromosomes were regarded as unable to divide mitotically (D`Amato 1964; Nagl 1981). For this reason, knowledge about the proportions of non-endocycling cells in explants used for *in vitro* polyploidizations is of peculiar interest for coming approaches in

midday flowers and other plants with polysomatic tissues. However, Galbraith et al. (1981) observed a deduplication of endoreduplicated to mitotically active 2C cells in tobacco cell cultures. Valente et al. (1998) further investigated the process of deduplication and revealed that *Nicotiana tabacum* cell nuclei divided amitotically into unequal daughter nuclei when auxins and cytokinins were applied simultaneously. By further amitotic divisions the resulting daughter cells reduced their DNA contents to a 2C level and afterwards re-entered mitosis. From these completely deduplicated cells Valente et al. (1998) regenerated normal shoots. Recent studies in insects and mammals showed that amitotic divisions of endoreduplicated cells often lead to severe genomic aberrations (e.g. Fox et al. 2010, also see the review of Fox and Duronio 2013). To our knowledge, however, almost nothing is known about the consequences of amitosis in endoreduplicative plant tissues.

In an evolutionary context, meiotic non-reduction and union of unreduced gametes are believed to occur much more frequently than somatic chromosome doubling, and therefore can be regarded as the driving forces in polyploid formation (Ramsey and Schemske 1998). In addition to their importance for species speciation and adaption (Ramsey and Schemske 1998), 2n gametes have also been discovered as valuable tools in breeding programs (Younis et al. 2014). In contrast to somatic chromosome doubling (e.g. through mitotic spindle inhibitors), the use of unreduced gametes can result in increased heterozygosity and allelic diversity (Bretagnolle and Thompson 1995). For this reason we aimed to test for the presence of unreduced pollen grains in our plant material. The most commonly applied methods to do so are morphological screenings (as 2n pollen grains were often found to be larger than reduced ones), progeny analysis of interploidy crosses, analysis of microsporogenesis (for a review see Bretagnolle and Thompson 1995) and, rarely, screening of 2n pollen by flow cytometry (Van Tuyl et al. 1989; Bino et al. 1990; Kron and Husband 2015).

The objectives of this study were - for the first time - (1) to estimate nuclear DNA contents of selected genotypes of the Aizoaceae genera *Lampranthus* and *Delosperma*, (2) to characterize endoreduplication patterns in different plant organs in these genera, and (3) to screen for unreduced male gametes.

Materials and Methods

Plant material and cultivation

Investigations were carried out on three genotypes of the genus *Lampranthus* (referred to as genotypes 1, 2 and 3 below) and on the species *Delosperma cooperi* (Figure 1). An additional *Delosperma* genotype (referred to as genotype 2 below) was included in genome size estimations and pollen nuclei analyses. The plants were propagated by cuttings and cultivated in peat/sand substrate (3:1) at 20 °C mean daily temperature. Relative humidity in the greenhouse was adjusted to 40-60 % by automatic ventilation. High pressure sodium lamps (Philips MASTER Agro 400 W) were used to ensure minimum daily photoperiods of 16 hours and for supplementary lighting when solar radiation fell below 25 klx. Manual irrigation and fertilization were applied with 0.1 % Hakaphos rot® (8 % N: 12 % P_2O_5: 24 % K_2O, Compo, Münster, Germany). Cotyledon samples, used to determine endoreduplication patterns, were taken from *in vitro* grown seedlings, which were cultivated at 23 °C and 40 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) in Murashige and Skoog (1962) medium containing 30 g/l sucrose. The medium was solidified with 3 g/l Gelrite® (Duchefa, Haarlem, Netherlands) and the pH value was adjusted to 5.8 using 0.1 M KOH. The seeds were derived from crosses, in which *Lampranthus* genotypes 1 and 2 were used as maternal parents. Prior to transfer to culture medium the seeds were surface sterilized in 1 % NaOCl solution (supplemented with a drop of Tween 20) for two minutes and rinsed three times (1, 2 and 5 minutes) in sterile deionised water.

Determination of organ-specific endoreduplication patterns

The proportions of cells with different C-values were determined in at least five repetitions for young leaves (5 mm maximum length), fully developed leaves, internode sections (second from shoot tip), petaloid staminodes (coloured flower leaves, Figure 1), sepals, roots as well as cotyledons and primary roots of one week old seedlings. Internode sections and sepals of *Lampranthus* genotypes 2 and 3 were not analyzed. Cotyledons and primary roots were exclusively analyzed in progeny of *Lampranthus* genotypes 1 and 2. The tissues were chopped with razor blades in Petri dishes (according to Galbraith et al. 1983) after adding 400 µl of a nuclei extraction buffer (Partec PreciseP, Münster, Germany). For separation of cell debris the sample suspensions were passed through 30 µm Celltric filters (Partec, Münster, Germany) two minutes after chopping. Subsequently the isolated cell nuclei were stained with 1 ml of DAPI (4´,6-diamidino-2-phenylindole) buffer (Partec PreciseP Staining Buffer, Münster, Germany) and analyzed flow cytometrically with a CyFlow Ploidy Analyzer (Partec, Münster, Germany). Signal amplifications (gain) were adjusted for each genotype and ranged between 500 and 600 V. In one measurement 2000-15000 nuclei were recorded, depending on the type of tissue. Following manual gating of peaks, the percentages of nuclei with different C-levels in the particular organ were calculated (based on the total number of recorded nuclei per sample). Mean percentages were averaged over the results of at least five analyzed samples per plant organ. In addition, `cycle values´ (indicating the mean number of endoreduplication cycles per nucleus) were calculated for each plant organ according to Barow and Meister (2003).

Observations on microsporogenesis and microgametogenesis

To gain insights into the formation of pollen grains, thirty flower buds of *Lampranthus* genotype 1 were collected in all stages of development (from barely visible to shortly before

anthesis). The buds were measured using a stereo microscope (Zeiss Stemi 2000 C) and the Zeiss AxioVision software and afterwards sorted according to their widths (2 - 7.2 mm). Anthers were excised from each flower bud, transferred to microcentrifuge tubes and fixed in ethanol/glacial acetic acid (3:1). Before microscopic analyses the fixation solution was replaced by DAPI staining buffer (Partec, Münster, Germany) and the air was evacuated from the samples for at least ten minutes. Subsequently, anthers were squashed on glass slides and their contents were inspected by fluorescence microscopy (AxioScope A1 equipped with filterset 02, 358 nm, Zeiss, Jena, Germany). Flower buds of *Delosperma* genotype 2 were examined in the same manner, but only in a late developmental stage (approx. 4.5 – 5 mm width). For observations of nuclei in pollen grains extracted from completely opened flowers of both genotypes, the grains were pre-treated by incubation in nuclei extraction buffer (Partec, Münster, Germany) for a few hours. In this way, an exine-covering exudate layer ("Pollenkitt"), which was autofluorescent and hindered microscopic insights, was dissolved. Afterwards the pollen grains were stained with DAPI as described above. Pollen diameters were determined using the Zeiss AxioVision software.

Estimation of genome sizes

As flow cytometric measurements of nuclei from endoreduplicated somatic tissues together with internal calibration standards resulted in overlapping of peaks, determinations of absolute nuclear DNA contents (pg/2C) were carried out on nuclei extracted from pollen tubes. Pollen of five to ten completely opened flowers of each genotype was collected in Petri dishes and dried for 24 hours at room temperature. Afterwards the pollen was transferred to Petri dishes containing Brewbaker and Kwack (1963) germination medium (solidified with 5 g/l Gelrite®) with a brush. Subsequently, media surfaces were screened for contaminations with anthers, filaments or other somatic debris under a stereo microscope. When detected, such contaminations were removed carefully by forceps. The inoculated dishes were sealed

with Parafilm® (Bemis Company, Neenah, USA) and placed at room temperature for 24 hours. In a next step the layer of pollen grains and tubes was carefully scratched from the surface of the germination medium with a razor blade and transferred into a further empty Petri dish. After adding 400 µl of the Partec nuclei extraction buffer the pollen tubes were co-chopped with a small amount of leaf tissue of a *Solanum lycopersicum* cultivar with known genome size (`Stupické Polní Rane´, 1.96 pg/2C, genebank Gatersleben acc. no. LYC 418). For *D. cooperi* the *Pisum sativum* cultivar `Viktoria, Kifejtö Borsó´ (9.07 pg/2C, genebank Gatersleben acc. no. PIS 630) was chosen as an internal standard, because measurements with *S. lycopersicum* resulted in partial peak overlapping. Ten minutes after chopping, the homogenates were passed through 30 µm Celltric filters (Partec) to remove ungerminated pollen grains and cell debris. Subsequently, 1 ml RNase-complemented propidium iodide buffer (AbsoluteP Staining Buffer, Partec, Münster, Germany) was added to each sample tube. The samples were kept on ice in complete darkness for two hours before the flow cytometric measurements. In each measurement the numbers of recorded nuclei ranged from 234 to 15492 for pollen nuclei (1C) and from 1127 to 19975 for nuclei of the calibration standards (2C). After manual gating of peaks nuclear DNA contents were calculated as follows

$$\text{Sample 2C value (pg DNA)} = \text{Reference 2C value x } \frac{\text{sample 1C mean peak position}}{\text{reference 2C mean peak position}} \text{ x 2}$$

Mean DNA contents were averaged over the results of four independent samples per genotype.

Flow cytometric and microscopic analyses of pollen nuclei

Flow cytometric analyses with the aim to verify the presence of unreduced male gametes were performed as described for genome size estimations with the following modifications: The analyses focused on *Lampranthus* genotype 1 and *Delosperma* genotype 2. At three different

dates pollen was extracted from 15 flowers per genotype and dispersed on solidified germination medium in at least three Petri dishes, respectively. After chopping of pollen tubes the nuclei suspensions were passed through 10 µm Celltric filters (Partec) and stained with 800 µl of DAPI buffer (Partec PreciseP Staining Buffer, Münster, Germany). To identify the C-levels of nuclei with different peak positions additionally somatic reference material (young leaves) was measured with the same signal amplification as applied for pollen samples. Based on the total number of recorded particles per measurement, the percentages of pollen nuclei with putatively different C-values were calculated. After flow cytometric analyses remnant nuclei suspensions were screened by fluorescence microscopy to clarify the cause for signals higher than 1C.

Statistical analysis

The percentages of non-endoreduplicated cells (2C) in different plant organs of each genotype were log-transformed and compared using Tukey tests (R-software version 3.0.2) to determine statistically significant differences ($\alpha = 0.05$).

Results

Determination of organ-specific endoreduplication patterns

Flow cytometric measurements as well as microscopic observations of sample suspensions revealed that all organs of the investigated midday flowers contained nuclei with up to five different C-levels (2C-32C; Figure 2) corresponding to increasing nuclei sizes and DNA contents. Non-endoreduplicative tissues were not detected. In the histogram shown in Figure 2 only four peaks (2C-16C) are displayed at their expected positions because the cytometer software collected all fluorescence signals higher than 230 units at the right side of the scale (small >16C peak). By reducing the signal amplification (gain) the 32C peak shifted to its

correct position, but then 2C and debris peaks could not be separated precisely for gating. As flow cytometry does not discriminate between 4C endocycling cells and those in mitotic G_2 or M phase, nuclei with a C-value higher than 2 were generally regarded as endoreduplicated below. In *Lampranthus* the lowest degrees of endoreduplication were detected in roots of greenhouse grown plants, which contained 23 % (genotype 2; cycle value: 0.26) to 29 % (genotype 3; cycle value: 0.31) cells with a DNA content between 4C and 16C (Figure 3; Suppl. Figure 1). In roots of *Delosperma cooperi* this percentage was slightly higher (34 %, 4C-16C, cycle value: 0.38; Figure 3). Contrary to roots of greenhouse grown plants, primary roots of *Lampranthus* genotype 1 seedlings were highly endoreduplicated (71 %, 4C-16C, cycle value: 1.16; Figure 3). In comparison to other organs, young leaves of all investigated plants contained relatively low amounts of endoreduplicated cells. These ranged between 17 % (*D. Cooperi*; cycle value 0.19) and 56 % (*Lampranthus* genotype 1; cycle value: 0.64). In fully developed leaves their proportion rose significantly up to 90 % (*Lampranthus* genotype 1; cycle value: 1.71; Figure 3). Cotyledon cells of one week old seedlings already ran through up to three or four endocycles. In these, high proportions of 74-87% of nuclei had a DNA content higher than 2C (cycle values: 1.59-1.89; Figure 3; Suppl. Figure 1). *Lampranthus* petaloid staminodes (cycle values: 1.55-2.37) and sepals (1.66) as well as *Delosperma cooperi* sepals (1.2) showed the highest percentages of endocycling cells (up to 95 % in *L.* genotype 2 staminodes and 80 % in *D. cooperi* sepals; Figure 3; Suppl. Figure 1).

Observations on microsporogenesis and microgametogenesis

After reaching a width of approximately 3 mm, flower buds of *Lampranthus* genotype 1 contained pollen mother cells undergoing meiotic prophase I. Up to a width of approximately 4 mm, all meiotic phases as well as tetrad formations were found, sometimes in the same flower bud. Aberrations from normal meiosis, indicating unreduced male gametes (e.g. formation of microspore dyads or triads), were not observed. During diakinesis and metaphase I nine bivalents became visible (Figure 4), implying a somatic chromosome

number of 2n = 18. Buds with a width of approximately 5 to 6 mm either exclusively contained polarized, vacuolated microspores or binuclear pollen grains. In buds shortly before anthesis (approximately 6.5 mm; staminode colour visible at bud tip) as well as in entirely opened flowers all grains have completed second pollen mitosis without exception. During the period of anther dehiscence, hydrated pollen grains distinctly varied in size. Measurements revealed diameters from 26 µm to 45 µm (Suppl. Figure 2a). In late developmental stages of *Delosperma* buds binuclear as well as trinuclear pollen grains were observed (Suppl. Figure 3). Binuclear grains were no longer detected in pollen extracted from flowers during anthesis. The fully developed grains had diameters ranging between 15 and 25 µm.

Estimation of genome sizes

The investigation revealed relatively low nuclear DNA amounts in all genotypes, ranging between 1.18 pg/2C (*Delosperma* genotype 2) and 3.68 pg/2C (*D. cooperi*; Table 1). The genome sizes of *Lampranthus* genotypes 2 and 3, which share many morphological traits, were determined to be equal (1.60 pg/2C). In measurements of propidium iodide stained pollen nuclei in the absence of a calibration standard, the flow cytometer displayed one main peak (1C) and, in case of *Lampranthus* genotype 1 and *Delosperma* genotype 2, a second much smaller peak corresponding to 2C DNA amounts (see also results of pollen nuclei analyses). Mean coefficients of variation (CVs) ranged between 6.7 and 7.6 in peaks of pollen nuclei, and between 4.6 and 6.0 in peaks of the tomato standard. CVs of the pea standard (applied for genome size estimation of *D. cooperi*) were slightly higher (approx. 6.3; Tab. 1).

Flow cytometric and microscopic analyses of pollen nuclei

Fluorescence microscopic analyses of pollen collected from opened flowers of all genotypes revealed that the grains are shed in a tricellular state (see above). Furthermore, observations on microgametogenesis in *Lampranthus* genotype 1 showed that the second pollen mitosis

took place before anthesis without exception. This information was essential for correct interpretation of data generated by flow cytometric pollen analyses, as generative nuclei of bicellular grains have 2C DNA contents and therefore would mimic unreduced gametes. Screenings of nuclei isolated from pollen tubes of *Lampranthus* genotype 1 and *Delosperma* genotype 2 resulted in the detection of particles corresponding to 1C, 2C and 3C DNA contents (Figure 5, Table 2). Although the two investigated genotypes exhibited varying diameters of pollen grains as well as differently sized sperm nuclei (Figure 6a), 2C proportions of up to 31 % of recorded particles could not be explained by presence of unreduced male gametes alone. Fluorescence microscopic observations of pollen nuclei suspensions revealed that the majority of nuclei were completely separated from each other. Nevertheless, a notable amount of sperm nuclei appeared as pairs, associated by structures which were not visualized through DAPI staining. Rarely, these sperm doublets were also linked to a vegetative nucleus (Figure 6c). Also in pollen tubes cultured *in vitro* for 24 hours most nuclei appeared separately, but occasionally sperm nuclei still shared the former generative cell wall (Figure 6b). In some cases, one of the sperm cells appeared to be bound to a remarkably stretched vegetative nucleus (Suppl. Figure 4). Pollen germination was observed in small grains as well as in those with exceptionally large diameters (Suppl. Figure 2b).

Discussion

Genome sizes

De Rocher et al. (1990) estimated the genome size of the annual Aizoaceae species *Mesembryanthemum crystallinum* flow cytometrically at 0.86 pg/2C. In a Feulgen microdensitometry approach the 2C DNA amount of *M. crystallinum* had already been estimated at 0.9 pg (Bennett and Leitch 1995). Although Soltis et al. (2003) declared genome sizes within the entire Aizoaceae family to be generally smaller than 1.4 pg/1C, to our

knowledge no published data on genome size estimations of midday flowers other than *M. crystallinum* were available so far. The nuclear DNA contents determined in *Lampranthus* and *Delosperma* were estimated for the first time and ranged between 1.18 and 3.68 pg/2C (Table 1). Across angiosperms, known genome sizes range between 0.13 pg/2C (*Genlisea margaretae* Hutch., Lentibulariaceae) and 304.4 pg/2C (*Paris japonica* Franch., Melanthiaceae) (Greilhuber et al. 2006; Pellicer et al. 2010). Hence, the classification of midday flowers as plants with small genomes is supported by the data obtained for the genotypes of this study.

With the exception of *Delosperma cooperi*, unfortunately no information about the breeding history of the investigated plant material was available. Nevertheless, *Lampranthus* genotype 1 shared growth and flower characteristics with the species *L. roseus* and genotypes 2 and 3 with *L. aureus* (Figure 1, e.g. described by Van Jaarsveld and De Pienaar 2004). In *Lampranthus* genotype 1, a nuclear DNA content of 2.36 pg/2C (Table 1) is distributed among 18 chromosomes, as nine bivalents were observed during microsporogenesis (Figure 4). This corresponds to the diploid chromosome set described for a number of species within the Ruschioideae subfamily (including *Delosperma sp.*) (Albers and Haas 1978) as well as for *M. crystallinum* (Adams et al. 1998). Unfortunately, efforts made by us to determine chromosome numbers also for the rest of the plant material were unsuccessful. Although cytometric data may in some cases only be loosely correlated with chromosome counts (Suda et al. 2006), we assume that the remaining *Lampranthus* genotypes were diploid as well because of their distinctly smaller genome sizes (Table 1). On the other hand, the investigated specimen of *D. cooperi* might have been polyploid, as its nuclear DNA amount (3.68 pg/2C) was more than three times higher than that of *Delosperma* genotype 2 (1.18 pg/2C). Tetraploid (Wulff 1944; Hartmann 1986; Hammer and Liede 1992) and even hexaploid specimens (Hartmann 1986) were repeatedly detected within species of Ruschioideae genera.

The problem of overlapping of sample and standard peaks due to endoreduplication in samples from somatic tissues could be solved to a great extent by the use of gametophytic nuclei from pollen tubes. Very high numbers of nuclei screened in single measurements constitute another advantage of the applied method. Principally, genome size estimations in the manner described are only reasonable if the DNA content of pollen nuclei remains constant during tube growth. In a microspectrofluorometry approach Friedman (1999) observed an increase of sperm DNA amounts to 1.75C in *Arabidopsis thaliana* pollen tubes. However, in other plant species producing trinucleate grains the DNA content of nuclei in pollen tubes was estimated at 1C (e.g. Hirano and Hoshino 2009). Also in the present study, comparisons to somatic reference material indicated that DNA contents of nuclei extracted from cultured tubes correspond to 1C (Figure 5). Conversely, the analyses also showed that the peaks at the very left in cytometric histograms obtained after measurements of somatic cells were indeed caused by 2C nuclei. In highly endoreduplicative plants such as orchids, 2C nuclei are often underrepresented and therefore overlooked in genome size estimations. Especially in such cases, the utilization of pollen nuclei represents a genuine alternative (Trávníček et al. 2015). Kron and Husband (2012) compared genome sizes estimated by using somatic and pollen nuclei in different plant species and only detected differences of less than three per cent. They postulated that structural differences of the analyzed types of nuclei could have been the reason for slightly divergent results.

Endoreduplication patterns

More than seventy years ago, Wulff (1940) discovered the occurrence of polysomaty in roots of various midday flower species, albeit the process of endoreduplication in plants was only poorly understood at that time. The majority of angiosperm species contain endoreduplicated cells, but in many cases the polysomaty is restricted only to certain tissues or cell types (e.g. embryo suspensor cells) (D`Amato 1984). However, the analysis of *M. crystallinum* by De Rocher et al. (1990) revealed, that cells with the original DNA content (2C) were

underrepresented in almost all differentiated plant parts. They found similar endoreduplication patterns in eight other succulent plants from different families and concluded that the phenomenon could be widespread among CAM plants with small genomes. Nagl (1976) already postulated a connection between distinct polysomaty and small genome sizes in various plant and insect species.

Because nuclear DNA contents and cell volumes are positively correlated in angiosperms (e.g. Jovtchev et al. 2006), endoreduplication facilitates the development of succulent, water-storing tissues in plants with small genomes. For this reason endoreduplication is regarded as a strategy for adaption to arid environments (De Rocher et al. 1990; Del Angel et al. 2006). However, Barow and Meister (2003) relativized the assumption of an exclusive correlation between genome size and highly expressed polysomaty. A statistical comparison of data collected for 54 plant species showed that the extent of endoreduplication primarily depends on the taxonomic position of a plant. In annual and biennial species endoreduplication was detected frequently. Less often the process was observed in perennials and it seems to be absent in woody plants (Barow and Meister 2003). Within the taxonomic order Caryophyllales, to which the Aizoaceae belong, the occurrence of endoreduplication was confirmed several times, e.g. within the families Cactaceae (De Rocher et al. 1990, Palomino et al. 1999, Del Angel et al. 2006, Sliwinska et al. 2009), Portulacaceae (Mishiba and Mii 2000), Amaranthaceae (formerly Chenopodiaceae) (Barow & Meister 2003, Kolano et al. 2009) and Caryophyllaceae (De Rocher et al 1990, Agullo-Antón et al. 2012).

Cells of *Delosperma* and *Lampranthus* genotypes investigated in the present study ran through up to four endocycles, as five different nuclei classes (2C-32C) were detected in their tissues. The mean number of endoreduplication cycles per nucleus (defined as`cycle value´ by Barow and Meister 2003) ranged from 0.19 in young leaves of *Delosperma cooperi* to 2.37 in petaloid staminodes of *Lampranthus* genotype III (Figure 3; Suppl. Figure 1).

As in *M. crystallinum* (De Rocher et al. 1990) and many other species (Barow and Meister 2003) the cotyledons of the investigated *Lampranthus* genotype showed a high degree of polysomaty (Figure 3). Whether endoreduplication in cotyledons simply enables faster growth through gained cell volumes or also the increased metabolic activity of endoreduplicated cells is of particular importance in this tissue, remains unanswered.

In the present study, roots of greenhouse grown plants turned out to contain relatively low proportions of endoreduplicated cells in comparison to other investigated plant parts (Figure 3; Suppl. Figure 1). In contrast, roots of *M. crystallinum* were found to be highly endoreduplicative (De Rocher et al. 1990). According to Barow and Meister (2003) endoreduplication conspicuously often occurs in plant species inhabiting extreme ecological niches and which therefore rely on fast development to survive. As annual desert plant *M. crystallinum* belongs to this group. Possibly, endoreduplication-facilitated root elongation (also see Hayashi et al. 2013) is less important for the perennial genera *Delosperma* and *Lampranthus* than for annual species. However, directly after seed germination fast root growth could be advantageous in their arid habitats. Therefore, the high degree of endoreduplication detected in primary roots of *Lampranthus* seedlings (Figure 3) might be considered as an adaption.

Aboveground, the most extreme endoreduplication patterns (up to 32C; max. cycle value: 2.37) were found in the pollinator attracting flower leaves (Figure 3; Suppl. Figure 1). In the survey of Barow and Meister (2003), cycle values similar to the maxima found in this study were only detected in stamens of *Spinacia oleracea* (2.30), whereas cycle values of petals in no account exceeded 1.12 (*Cucurbita pepo*). Continuous DNA replication might not only accelerate the expansion of petaloid staminodes, but also contributes to their relatively large sizes in midday flowers. Only marginally different endoreduplication patterns were found in greenhouse-cultivated and *in vitro* grown tissues of *D. cooperi* (Braun and Winkelmann

2015), suggesting that the process is not influenced by environmental conditions like relative humidity or light intensity. Also in the highly endoreduplicative (2C-16C) Cactaceae species *Mammillaria san-angelensis* no deviations from normal endoreduplication patterns, even in the presence of phytohormones, were observed after long term *in vitro* culture (Palomino et al. 1999). Galbraith et al. (1991) described developmentally regulated endoreduplication for *A. thaliana*. They discovered that the organ-specific endoreduplication patterns of plants cultivated under long- and short-day conditions showed no differences.

As described above, the proportions of non-endoreduplicated cells differed significantly among plant organs of midday flowers, suggesting that the success of shoot regeneration in *in vitro* polyploidization approaches might be correlated with the type of chosen explants. Coming experiments will show, whether deduplications of endocycling cells (as described by Galbraith, 1981; Valente et al. 1998) also occur in the investigated genotypes and which consequences for *in vitro* regenerants can be expected.

At this time, the general effect of chromosome doubling through spindle inhibitors in endoreduplicative plants remains largely uncertain. Mishiba and Mii (2000) successfully polyploidized *Portulaca grandiflora* using colchicine. The endoreduplication patterns in the generated tetraploid plants were found to be much weaker than those of the wild-type.

Screening for unreduced male gametes

As DNA contents and volumes of pollen grains were generally thought to be positively correlated (Bretagnolle and Thompson 1995), considerably varying pollen diameters in some of our genotypes provided a first indication for spontaneous production of unreduced male gametes. Nevertheless, simple measurements of pollen grains were not appropriate for ploidy level classifications, because their diameters were almost normally distributed between the smallest and largest determined sizes (Braun and Winkelmann 2015). This problem has already been described for other plant species. Aguilar-García et al. (2012) failed to detect 2n

gametes in the Cactaceae species *Pachycereus weberi* morphologically, because pollen diameters ranged between 5 and 950 µm. Moreover, Jansen and Den Nijs (1993) detected overlapping diameters of haploid and diploid *Lolium perenne* pollen grains.

For this reason we decided to analyze pollen nuclei flow cytometrically, a technique that has only been rarely applied for the detection of unreduced gametes (e.g. by Van Tuyl et al. 1989, Bino et al. 1990, Kron and Husband, 2015). However, in comparison to alternative detection methods, like analysis of microsporogenesis or progeny analysis of directed hybridizations, flow cytometry is less time-consuming and labour-intensive (Bretagnolle and Thompson, 1995). Besides high amounts of haploid 1C nuclei, notable percentages (up to 31 %; Table 2) of nuclei corresponding to 2C DNA contents were detected in measurements of Aizoaceae pollen. Generative nuclei in the G_2 phase of the cell cycle can be excluded as potential source for 2C signals, because the second pollen mitosis in *Delosperma* and *Lampranthus* was found to generally take place before anthesis. Moreover, it appeared very unlikely that unreduced gametes were the primary cause for the detection of 2C signals, because (1) such high rates of spontaneous production have only been rarely observed in previous studies (Bretagnolle and Thompson 1995) and (2) a third class of particles corresponding to 3C DNA amounts was identified by our measurements. Finally, microscopic analyses of sample suspensions indicated that 2C as well as 3C signals could be explained by partly persistent connections between pollen nuclei.

The physical connection between both sperm cells and the linkage of one sperm cell to the vegetative nucleus inside pollen grains and tubes is referred to as male germ unit (MGU), which was first described by Russell and Cass (1981) in *Plumbago zeylanica*. In *Nicotiana tabacum* the two sperm cells are structurally connected by a shared cross wall containing fibrils, tubules and callose (Yu et al. 1989; Yu et al. 1992) The linkage of sperm doublets to the vegetative nucleus is maintained by a cytoplasmic projection of one sperm cell, which

notably extends during gamete transport in pollen tubes (McCue et al. 2011). In flow cytometric analyses of *Alstroemeria aurea* pollen nuclei including prior microcapillary separation of particles in isolates, Hirano and Hoshino (2009) demonstrated for the first time that measurements of sperm doublets result in 2C and entire MGUs in 3C signals. The stability of MGUs (particularly sperm doublets) in nuclei extraction buffer presumably can be ascribed to the distinctive cytoskeleton of sperm and generative cells. It consists of microtubule bundles which form basket-like arrays along the cell axis and terminate in cellular extensions (Palevitz and Liu 1992; Southworth and Cresti 1997). The presence of actin-containing microfilaments in sperm cells (e.g. assumed by Taylor et al. 1989) was disproven by Palevitz and Liu (1992). Recently, Kron and Husband (2015) took up the issue of delusive sperm doublets in flow cytometric 2n screenings. They presented a possibility for "doublet correction" in flow cytometric data, which is based on pulse analysis. As pulse analysis is only well applicable if the nuclei of interest are spherical and homogeneous in shape (Kron and Husband 2015), it is probably not appropriate for analysis of Aizoaceae pollen. Observed by fluorescence microscopy, vegetative nuclei were highly heterogeneous and sperm nuclei had an elliptical shape after extraction from pollen tubes. Still there are some options remaining to verify the presence of unreduced male gametes in the investigated plant material at last. One of them could be a microspectrofluorometry approach to compare DNA contents of sperm nuclei in pollen tubes as conducted by Friedman (1999). As another possibility, analyses on microsporogenesis could be intensified. In a few studies it was proven that polyploid pollen mother cells led to the formation of unreduced male gametes (Lelley et al. 1987, Falistocco et al. 1995, De Storme and Geelen 2013).

Finally we can conclude that, aside from *M. crystallinum* (De Rocher et al. 1990), pronounced endoreduplication is also a feature of midday flowers in the subfamily Ruschioideae. The genome sizes of the investigated Aizoaceae genotypes were found to be rather small. Regarding the difficulties in estimating genome sizes of endoreduplicative plants flow

cytometrically, measurements of pollen nuclei together with an internal standard turned out to be a convenient solution to the problem. On the other hand, flow cytometric screenings for unreduced gametes entail the risk of false-positive results due to persistent male germ units. Thus, when searching for pollen with the somatic chromosome number, flow cytometric data as well as increased grain diameters should be treated with caution.

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Tables

Table 1. Nuclear DNA contents estimated by flow cytometry using pollen nuclei

Genus	Genotype	Genome size (pg/2C ± SD)	Mean CVs ± SD Sample	Standard[a,b]
Lampranthus	1	2.36 ± 0.02	7.6 ± 0.3	5.7 ± 0.4
	2	1.60 ± 0.04	7.1 ± 1.0	6.0 ± 0.9
	3	1.60 ± 0.03	6.7 ± 1.1	5.7 ± 0.5
Delosperma	*cooperi*	3.68 ± 0.08	7.4 ± 0.2	6.3 ± 0.3
	2	1.18 ± 0.02	7.2 ± 0.9	4.6 ± 0.8

CVs = coefficients of variation of sample and calibration standard peaks; SD = Standard deviations of four replicates; [a]*Solanum lycopersicum* `Stupické Polní Rane´ (1.96 pg/2C), [b]*Pisum sativum* `Viktoria, Kifejtö Borsó´(9.07 pg/2C; applied for the genome size estimation of *D. cooperi*)

Table 2. Percentages of detected particles with fluorescence intensities corresponding to different C-values of pollen nuclei

		Date 1 % ± SD	Date 2 % ± SD	Date 3 % ± SD
***Lampranthus* I**	**1C**	85.6 ± 2.6	85.7 ± 3.1	91.1 ± 2.6
	2C	10.8 ± 1.3	9.4 ± 1.5	7.5 ± 2.0
	3C	3.6 ± 1.8	4.9 ± 1.7	1.4 ± 0.7
***Delosperma* II**	**1C**	63.2 ± 4.2	94.4 ± 1.5	93.6 ± 0.4
	2C	30.7 ± 3.1	5.5 ± 1.6	6.4 ± 0.4
	3C	6.0 ± 1.3	0.1 ± 0.2	0.0

Dates of analyses were 18 Feb., 25 Feb. and 19 March 2014 for *Lampranthus* and 2 April, 17 July and 30 July 2014 for *Delosperma*. Mean percentages were calculated from at least three independent repetitions; SD = Standard deviation

Figures

Figure 1. Flowers of the analyzed plants. (A-C) *Lampranthus* genotypes 1-3, (D) *Delosperma cooperi*

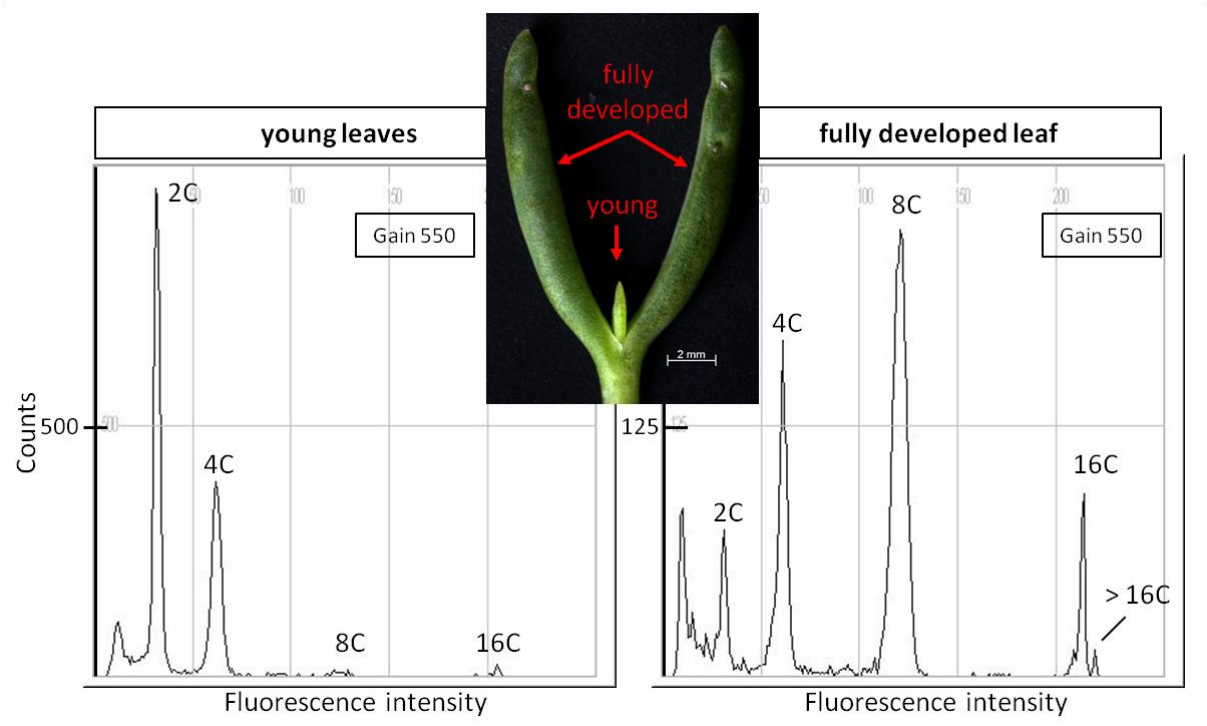


Figure 2. Flow cytometric histograms resulting from analyses of young and fully developed *Lampranthus* genotype 1 leaves. Peaks at the very left were caused by cell debris and/or extranuclear DNA.

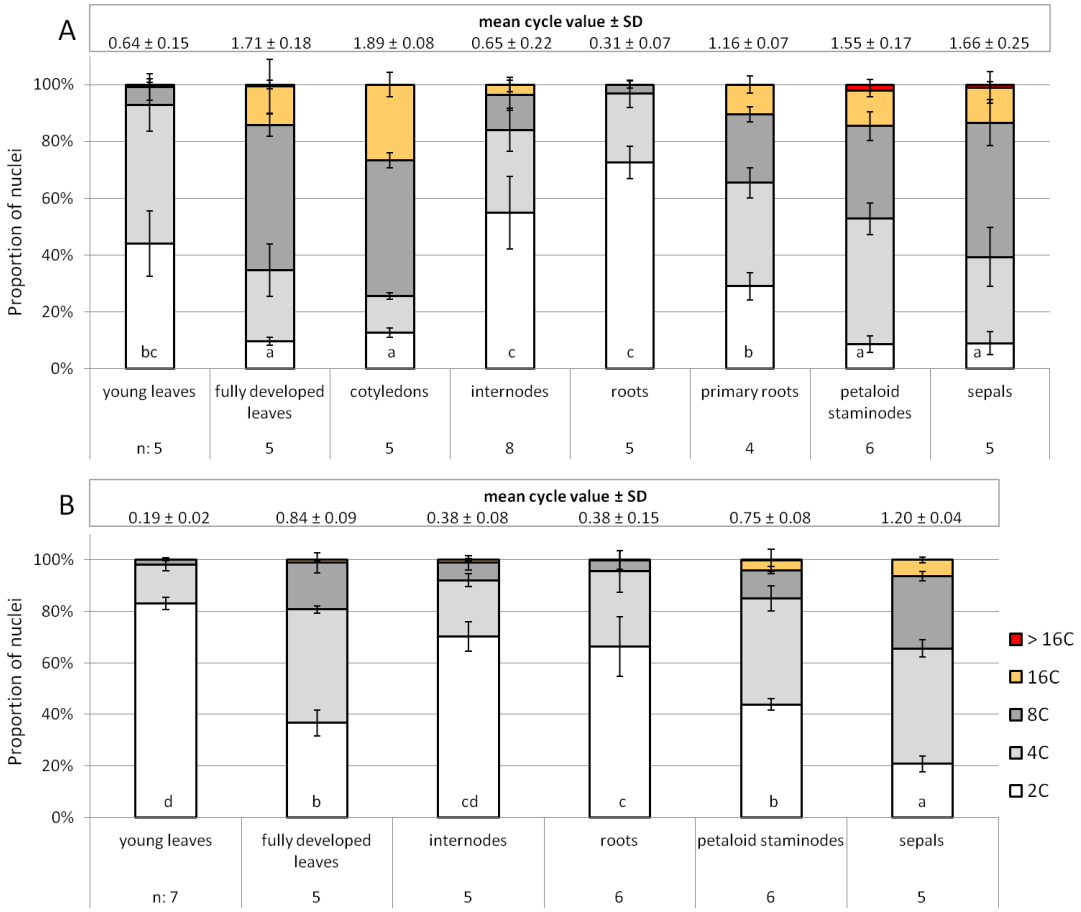


Figure 3. Cycle values and proportion of cell nuclei with different C-levels in various tissues of *Lampranthus* genotype 1 (A) and *Delosperma cooperi* (B). n = number of analyzed samples, I = SD, different letters denote statistically significant differences between organs regarding proportions of non-endoreduplicated (2C) cells (Tukey test; $\alpha = 0.05$).

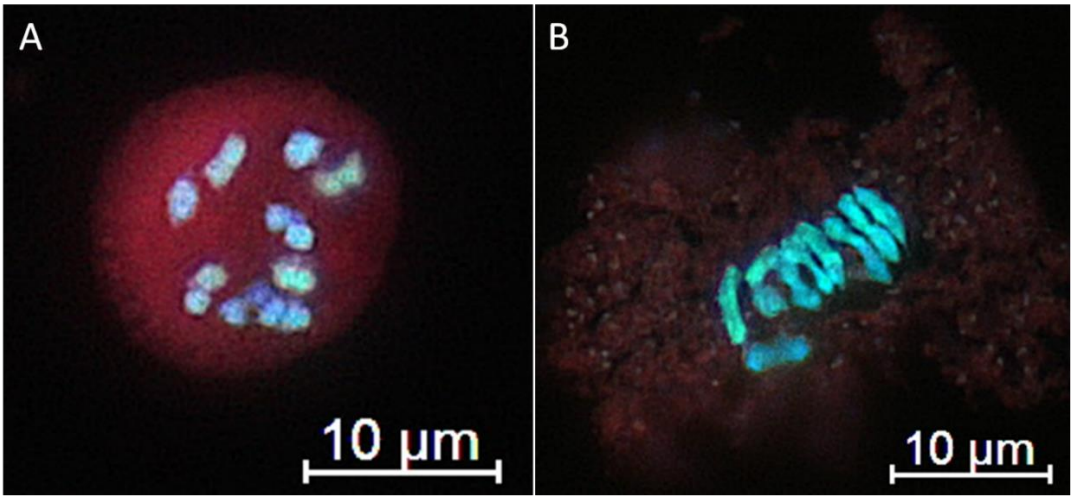


Figure 4. DAPI-stained meiotic (A) diakinesis and (B) metaphase I chromosome bivalents (9) of *Lampranthus* genotype1.

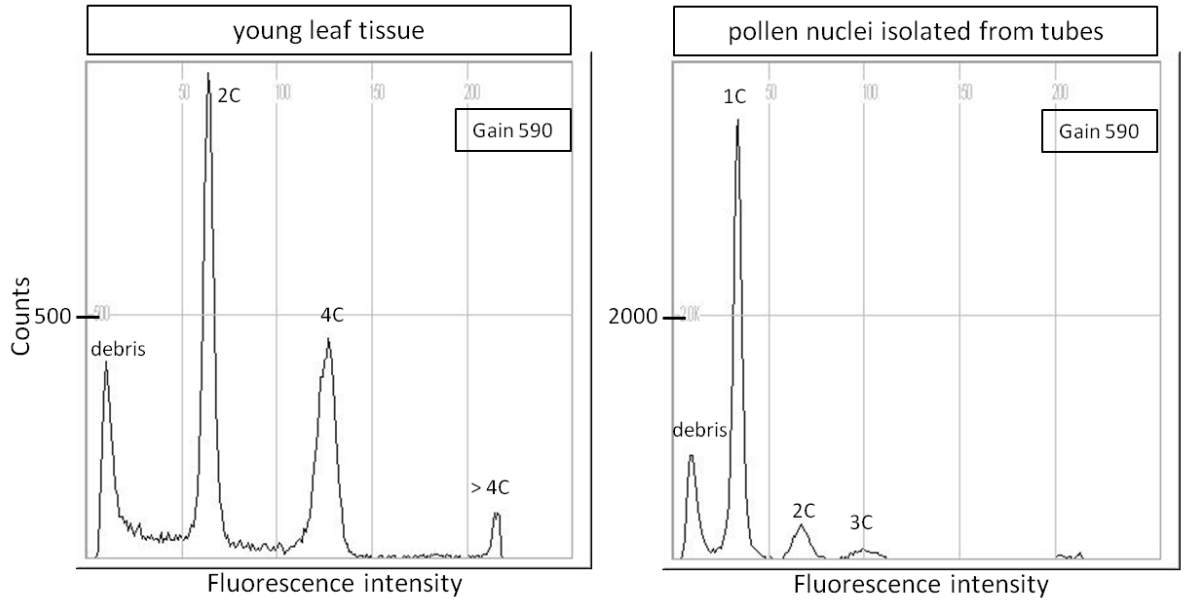


Figure 5. Flow cytometric histograms resulting from analyses of somatic reference material and nuclei isolated from *Lampranthus* genotype 1 pollen tubes.

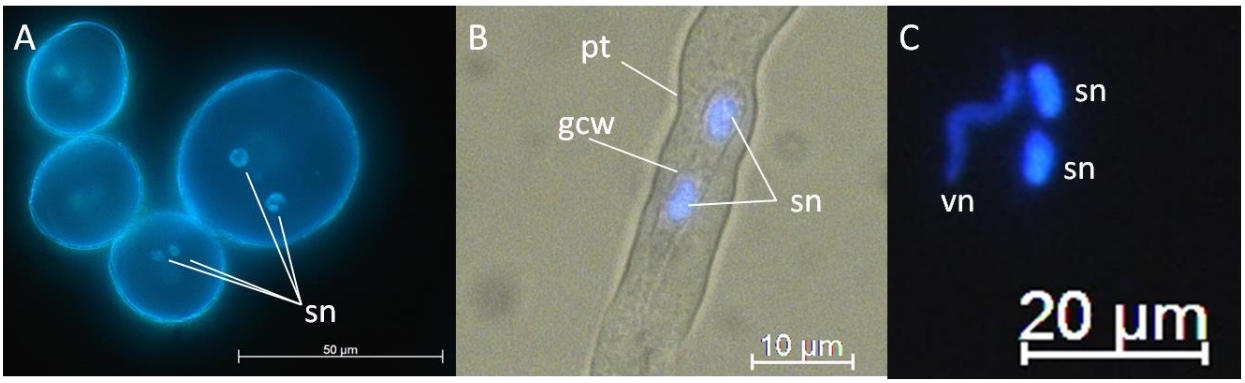
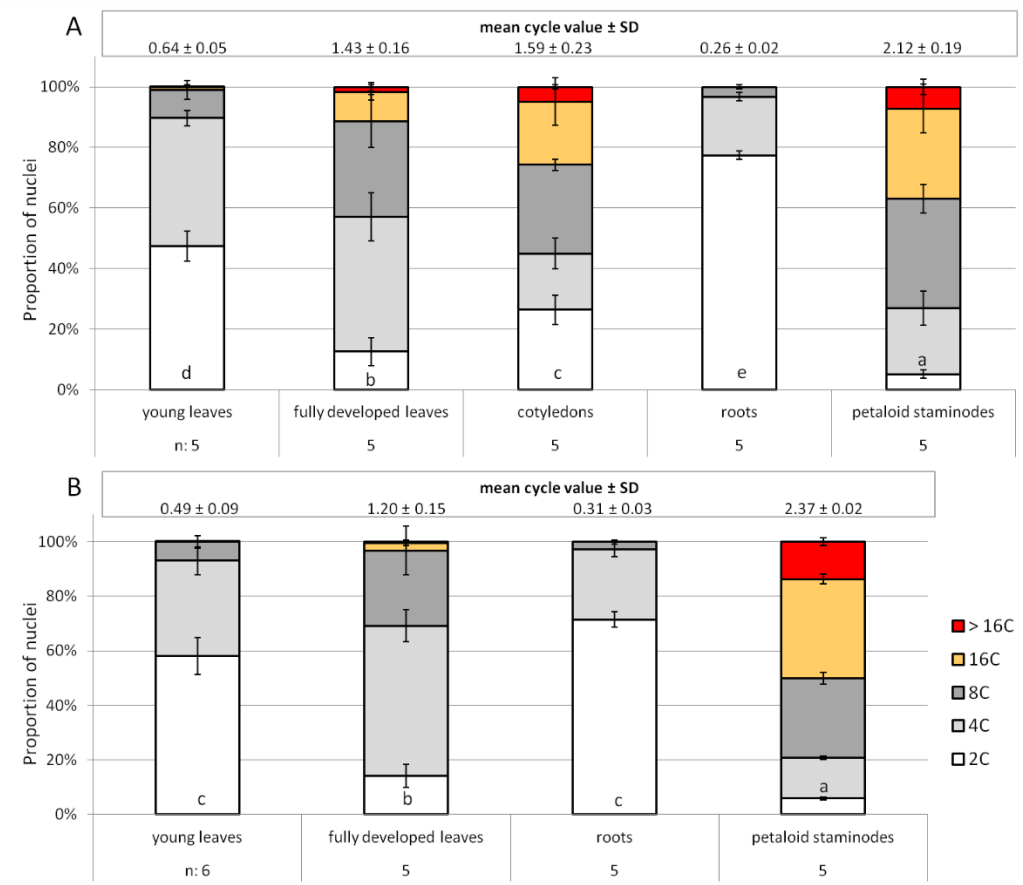
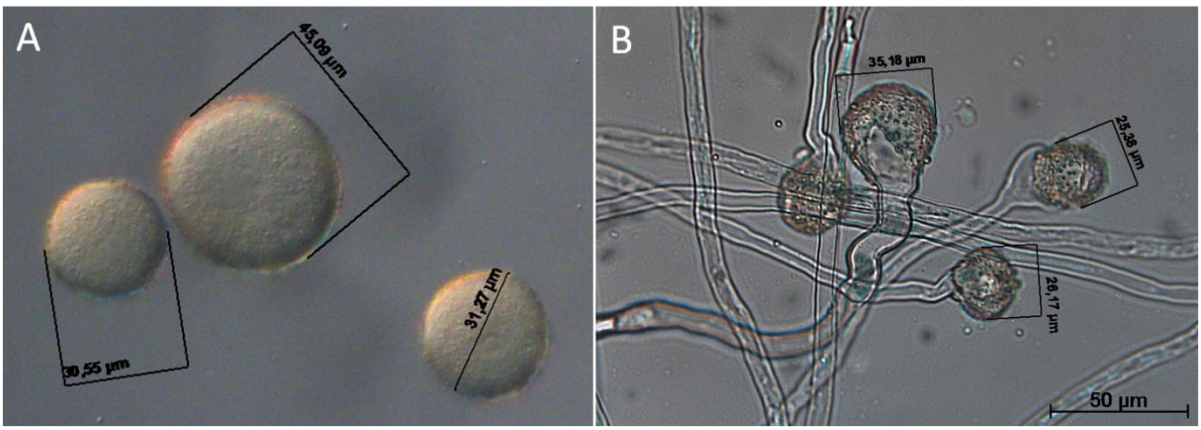


Figure 6. Micrographs of *Lampranthus* genotype 1 pollen grains and nuclei following DAPI staining. (A) Varying diameters of grains and sperm nuclei; (B) Sperm nuclei doublet enclosed by the generative cell wall in an *in vitro* grown pollen tube; (C) Intact male germ unit (MGU) in a nuclei suspension prepared for flow cytometric analysis. Abbreviations: sn, sperm nucleus; vn, vegetative nucleus; gcw, generative cell wall; pt, pollen tube.

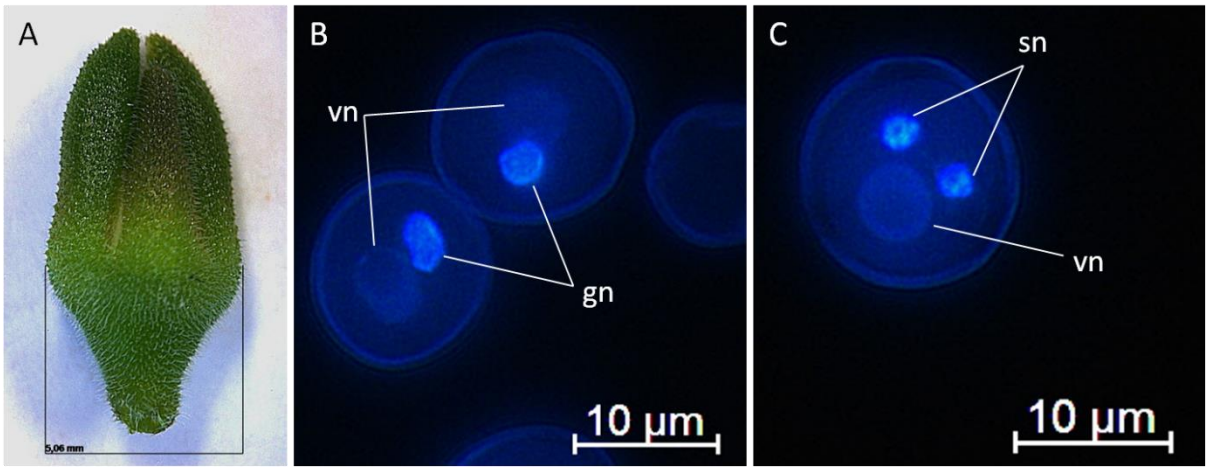
Additional files



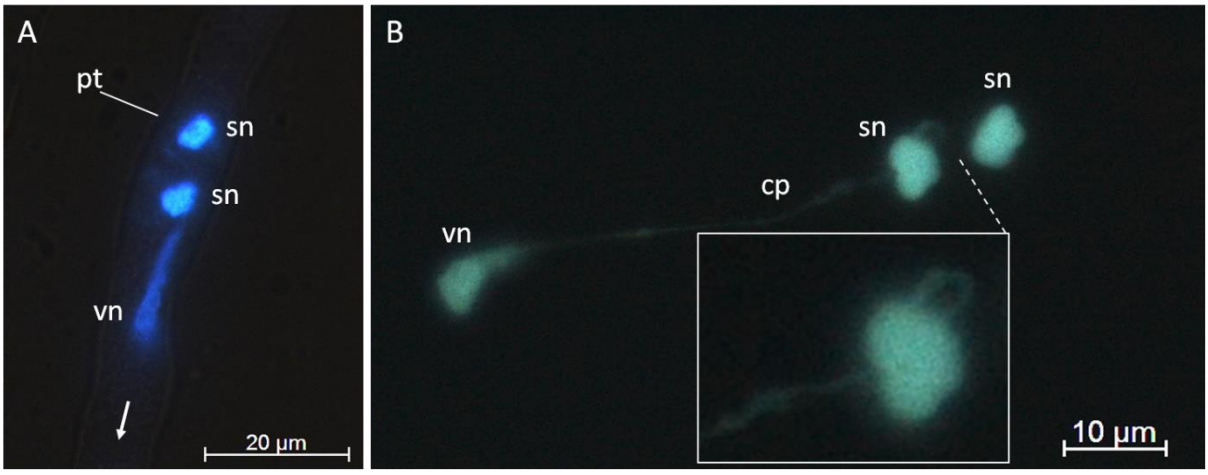
Supplemental Figure 1. Cycle values and proportion of cell nuclei with different C-levels in various tissues of *Lampranthus* genotype 2 (A) and 3 (B). n = number of analyzed samples, I = SD, different letters denote statistically significant differences between organs regarding proportions of non-endoreduplicated (2C) cells (Tukey test; $\alpha = 0.05$).



Supplemental Figure 2. Varying diameters of completely hydrated *Lampranthus* genotype 1 pollen grains (A) and Pollen tubes emerging from differently sized pollen grains *in vitro* (B).



Supplemental Figure 3. *Delosperma* genotype 2 flower bud shortly before anthesis (A), which contained binuclear (B) as well as trinuclear (C) pollen grains. Abbreviations: sn, sperm nucleus; vn, vegetative nucleus; gn, generative nucleus.



Supplemental Figure 4. DAPI-stained male germ units of *Lampranthus* genotype 1 in (A) an *in vitro* grown pollen tube (the arrow points towards the pollen tube tip) and (B) isolated from a bursted pollen tube on germination medium; detail: sperm nucleus and the cytoplasmic projection of the sperm cell. Abbreviations: sn, sperm nucleus; vn, vegetative nucleus; pt, pollen tube; cp, cytoplasmic projection.

6. Conclusions and Outlook

The results obtained in the different studies were already discussed extensively in the four article manuscripts. This chapter intends to summarize the main findings and conclusions and to emphasize some additional aspects. Moreover, an outlook for follow-up studies and application possibilities in midday flower breeding programs are given.

6.1 Factors controlling flowering in three Aizoaceae genera

Most genotypes of different Aizoaceae genera, which were cultivated under stable photoperiodic and climatic conditions in a greenhouse at the Leibniz Universität Hannover, only formed low numbers of flowers in an unpredictable manner. As flowering in South African ecosystems is known to occur synchronized and limited in time (e.g. Le Roux et al., 1989; Cowling et al., 1999), cues which naturally induce flowering were obviously missing in the marginally varying greenhouse environment. However, the missing cues appeared to be facultative in the majority of genotypes, because otherwise they would not have formed flowers at all. The autonomous pathway of flower induction, which is best described in *Arabidopsis thaliana*, enables flowering even under non-inductive conditions by downregulation of the floral repressor *FLC* (Srikanth and Schmid, 2011). Presumably, endogenous regulators comparable to those involved in the deciphered autonomous pathway were responsible for the nonuniform appearance of flowers in the greenhouse. Future molecular analyses could reveal if orthologues of the known regulators are present in Aizoaceae taxa.

Photoperiod, which was initially considered as a potential parameter, appeared not to play an important role for flower induction in the investigated genotypes (Chapter 2). Taking account of other studies on flowering of South African plant species (e.g. Van Rooyen et al., 1991; Adams et al., 1998; Dreyer et al., 2006; Ehrich et al., 2009, 2010; Thompson et al., 2011), photoperiodic responsiveness seems generally not to be widespread in regions where midday flowers occur. However, the formation of unusual small flowers and the abortion of buds in the short-day variants (Chapter 2) indicated that the received daily light integrals had an impact on the development of flowers after induction. The analyses of aboveground plant parts subsequent to the photoperiod experiment revealed that night interruptions given after short days increased the dry matter content of plants. This phenomenon has previously been described in other plant species, but the physiological reasons behind remain unclear (Adams and Langton, 2005). However, the positive impact of low-energy night interruptions should be verified in more extensive future experiments, because it might be an effective and cost-saving tool for quality improvements in the cultivation of midday flowers. Furthermore, impacts of light quality on flower formation and plant growth should be analyzed in future approaches, e.g. by using light sources with defined emission spectra (e.g. LEDs).

With due regard to the main flowering seasons in the natural habitats of the investigated genera (e.g. Le Roux et al., 1989; Struck, 1994; Hartmann, 2001), temperature was chosen as further potential flower-inducing factor. The experiment conducted thereupon revealed that the formation of flower buds in *Lampranthus* was promoted by low mean daily temperatures (14 °C), while *Delosperma* tended to develop more flowers at 20 °C. In Namaqualand, a South African winter-rainfall region with climatic conditions comparable to those in the natural habitats of *Lampranthus* and *Cephalophyllum* (Hartmann, 2001; Peel et al., 2007), the onset of flowering in several Aizoaceae species mainly depends on the time point of the first temperature drop in autumn (March/April) (Struck, 1994). Because the flowering period in Namaqualand begins no earlier than July or August (Struck, 1994)

and considering the late, but abrupt formation of many flower buds in the conducted experiment, a facultative cold requirement of the investigated *Lampranthus* genotypes is conceivable. However, after induction, the development of flowers was accelerated by higher temperatures. Further investigations involving midday flowers of other genera are needed to characterize the temperature requirements for flower induction and subsequent development in more detail. These should include histological analyses to determine the exact time of induction. Especially plants that show flexibility in temperature responses (such as the investigated *Cephalophyllum* and *Delosperma* genotypes) might be well suited for coming breeding approaches. If available, future approaches should also include hybrids obtained from cross-combinations of taxa with differing temperature requirements, as those might exhibit altered flowering patterns. Furthermore, the impact of water availability on flower formation and morphological traits should be considered in future experiments, as the growing periods in South African ecosystems are highly influenced by precipitation patterns (Le Roux et al., 1989; Struck, 1994; Cowling et al., 1999). In addition, the physiological and genetic mechanisms behind diurnal petaloid staminode movement should be investigated, as the full ornamental potential of most Aizoaceae taxa is only unlocked during flower opening.

6.2 The mechanisms of self-incompatibility in *Lampranthus* and *Delosperma*

The question whether a plant of interest is able to reproduce via seeds after self-fertilization is of particular importance for plant breeders. On the one hand, self-compatibility can be desirable in certain approaches (e.g. for generating inbred lines), but on the other hand, it is awkward when cross-pollinations are conducted to generate hybrid progeny. In the latter case, maternal flowers need to be emasculated before pollen gets in contact with the stigma. In plants that exhibit protandry, just as the investigated midday flowers (see also Braun 2012), emasculations can be laborious and time-consuming, because the male flower parts must be removed before anthesis. Although midday flowers were often regarded as largely self-sterile (e.g. Schwantes 1916, 1954; Brown, 1920; Van Jaarsveld and De Pienaar 2004), partial or full self-compatibility has been observed in various taxa (e.g. Hammer and Liede, 1990; Hammer, 1995, 2004; Suehs et al., 2004; Schröder, 2008). For this reason, the processes occurring after directed self-pollinations were analyzed through observations of pollen tube growth *in situ* (Braun 2012), observations of early embryogenesis using DIC microscopy and evaluation of capsule and seed development (Chapter 3). Seeds were neither formed in *Delosperma* nor in *Lampranthus* after self-fertilizations. In *Lampranthus*, however, pollen tubes reached the micropyles of ovules after self-pollinations and in one genotype some pre-globular embryos were observed. Thus, fertilization failures or postzygotic barriers caused the absence of seed formation in this genus. Whether the investigated *Lampranthus* genotypes are completely self-sterile remains unclear, because the principles of late-acting self-incompatibility (LSI) are only poorly understood (Gibbs, 2014). The occasional observation of embryos in one *Lampranthus* genotype furthermore raises the question of whether these could have formed apomictically. Apomictic embryos generally develop from unreduced cells of the female gametophyte or sporophyte. The majority of species found to reproduce asexually via seeds exhibit pseudogamy, which requires fertilization of the central cell for endosperm development (Grossniklaus et al. 2001). Facultative agamospermy among midday flowers has so far only been described in the genus *Capobrotus* (Vilà et al. 1998; Suehs et al. 2004) and underlying mechanisms have not been studied.

Because the pollen tube growth in *Delosperma* already stopped on the stigma surface and swollen ovules or embryos were not detected, the self-incompatibility mechanism in this genus appears to be S-locus-controlled (see also Takayama and Isogai, 2005). In other studies, at least some species of *Delosperma* were found to be self-compatible before (Hammer and Liede, 1990, Schröder, 2008). For

this reason, the conclusions drawn from the experiments on self-compatibility in this thesis are only valid for the investigated genotypes. However, as the DIC microscopy technique even allowed the detection of pollen tubes in the proximity of ovules, the question of self-compatibility in other genotypes can easily be clarified. For breeding approaches in midday flowers self-sterility is advantageous, because it obviates the need for flower emasculations and ensures that formed seeds have a hybrid state.

6.3 Barriers to intra- and intergeneric hybridizations

The high germination rates of pollen grains determined in most genotypes indicated that lacking pollen viability was definitely not the cause for hybridization failures. Generally, pollen management appears to be a simple task in midday flower breeding, because storage of pollen for several days at room temperature and for longer periods at -20 °C did not result in drastic loss of viability (Braun, 2012).

The analyses of pollen tube growth and early embryogenesis following intra- and intergeneric cross-pollinations revealed that the required molecular cues for pollen tube guidance are congruent in the investigated *Delosperma* and *Lampranthus* genotypes (Chapter 3). However, failures of pollen tube reception and fertilization as well as deviations from normal embryogenesis indicated that these processes are regulated differently in the two genera. As the absence or low expression of prezygotic hybridization barriers increases the chance to obtain hybrid progeny in breeding programs, future experiments should verify whether similar observations can be made after cross-pollinations of other Aizoaceae taxa. To estimate the existence of alternative pollen tube guiding signals and the time point of their evolution, cross-pollinations should be performed within and between different tribes and subfamilies. Screening for orthologues of genes (e.g. encoding CRPs) that are known to be involved in the molecular crosstalk during pollen tube guidance and reception (e.g. Kessler and Grossniklaus, 2011; Dresselhaus and Franklin-Tong, 2013) could reveal whether the mechanisms in midday flowers are similar to those found in model plants. Furthermore, the identification of specific molecular signals could allow the prediction of prezygotic barriers in breeding programs.

Although aberrations from normal embryogenesis were detected early after combinations of incompatible genotypes, the reasons for the absence of seed development remain unclear. Histological analyses of fertilized ovules at later developmental stages by microtome sectioning could illustrate when exactly embryogenesis is disrupted and whether the abortion can be ascribed to endosperm degeneration. This information could be helpful for the development of embryo rescue techniques because the frequency of success often increases with age and size of the isolated ovules or embryos (Winkelmann et al., 2010). Furthermore, analysis of the complex ovary gland exudates in the Aizoaceae (e.g. Kristen et al., 1980; Kristen and Liebezeit, 1980) might facilitate the development of optimized nutrient media for future embryo rescue approaches.

Unbalanced chromosome sets were found to be a major cause for hybrid sterility (Rieseberg and Carney, 1998) as well as for deficient endosperm development (e.g. Nishiyama and Inomata, 1966). Therefore, divergent ploidy levels of cross-pollinated genotypes could have been responsible for both, embryo abortion and lacking pollen viability in some hybrid combinations. Unfortunately, chromosome counts in root tip cells were unsuccessful, mainly due to the small size of mitotic cell nuclei. The chromosome number of one *Lampranthus* genotype was successfully determined during analysis of microsporogenesis (Chapter 4). This genotype had 18 chromosomes, which corresponds to the diploid chromosome set described in the Ruschioideae subfamily (Wulff, 1944; Albers and Haas, 1978; Hartmann, 1986; Hammer and Liede 1992) and in *Mesembryanthemum crystallinum* (Adams et al., 1998). Because this plant showed the highest genome size of all cross-pollinated genotypes

(2.36 pg/2C), the rest of the plant material was assumed to be diploid as well. Thus, rather than diverging ploidy levels, the relatively high variation in genome sizes (1.18 – 2.36 pg/2C) could have influenced the hybridization success negatively. Diverging genome sizes of the cross-pollinated genotypes could have disturbed the genomic ratio of 2m:1p, which is usually required for normal endosperm development (Haig and Westoby, 1991). Whether uneven ratios of paternal DNA have been responsible also for the aberrant embryonic development after intergeneric crosses is uncertain and could be verified by histological analyses after reciprocal cross-combinations. By the current state of knowledge, the genomes of pollen and seed parents do not contribute equally to embryonic patterning during the first days after fertilization. In *Arabidopsis*, a parent-of-origin effect was for example detected in zygote elongation and suspensor development, both regulated by the *YODA* mitogen-activated protein kinase pathway. Paternal *SHORT SUSPENSOR* (*SSP*) transcripts in sperm cells are transported to egg and central cells, where their protein product is required to activate the *YODA* pathway (Bayer et al. 2009). On the other hand, Vielle-Calzada et al. (2000) found numerous paternal alleles to be silenced during early embryogenesis and endosperm development. According to Ng et al. (2012), the reactivation of transposable elements (TEs) in hybrid embryos and endosperm failures are highly influenced by the dosage and divergence of small maternal RNAs.

However, the most abundant postzygotic barriers observed after seed germination in this thesis were albinism and/or low hybrid vigor. As up to now no means to overcome these barriers exist, further embryo rescue approaches in the respective genotypes are inauspicious. Instead, other genotypes should be included in coming crossbreeding experiments.

While almost no incompatibilities were observed after intrageneric hybridizations of *Delosperma*, the success rate following cross-combinations in *Lampranthus* and intergeneric hybridizations were rather low. The hybrid state of plants that survived was confirmed by AFLP analysis, which is reliable and does not require prior sequence data (Vos et al., 1995). However, as in other succulent plants (e.g. Barnwell et al., 1998), isolation of DNA for molecular analyses turned out to be a difficult task in the investigated midday flowers. DNA isolation using prepared kits (e.g. by the manufacturers Macherey-Nagel, Qiagen and MoBio) resulted in very low yields, while DNA obtained by the methods according to Dellaporta et al. (1983) and Dorokhov and Klocke (1997) had low purity and integrity. Finally, DNA samples with adequate quality for AFLP analysis were obtained by applying the method of Doyle and Doyle (1987), which was relatively time-consuming and required the application of hazardous chemicals.

The confirmation of two viable intergeneric hybrids demonstrated that hybridization barriers between *Delosperma* and *Lampranthus* can be overcome. This insight makes breeding approaches in the Aizoaceae more promising and furthermore suggests that leaking reproductive isolation mechanisms might constitute one explanation for the fast diversification of the plant family. Interestingly, calculations revealed that the obtained intergeneric hybrids showed only low genetic similarity to the paternal *Lampranthus* genotype. Furthermore, the genome size of one intergeneric hybrid (~ 1.2 pg/2C) was found to be much smaller than that of the pollen donor (2.36 pg/2C). According to Davies (1974), uniparental chromosome elimination in interspecific hybrids frequently occurs during the first days of embryogenesis. If desired, the fate of paternal chromosomes could be clarified by genomic *in situ* hybridization (GISH) (e.g. Schwarzacher et al. 1989) or marker analysis (e.g. Fulcher et al. 2015).

While seeds obtained from intrageneric *Delosperma* crosses mostly showed high viability and germinated shortly after transfer to the *in vitro* medium, the germination rates of *Lampranthus* seeds (and ovules) were generally low and most seeds germinated after several weeks. In addition to the abovementioned postzygotic barriers, low germination rates in *Lampranthus* could possibly be

ascribed to seed dormancy. Physiological seed dormancy has been previously described in the Aizoaceae (Baskin and Baskin, 1998; Finch-Savage and Leubner-Metzger, 2006). According to Van Jaarsveld and De Pienaar (2004), after-ripening of seeds for more than one year increased germination rates in several midday flower species. Esch (2016) determined significantly higher germination rates in *Lampranthus* seeds that were previously stored at room temperature for nine months than in freshly harvested seeds. Studies on seed dormancy in the annual midday flower *Mesembryanthemum nodiflorum* revealed that the expression of dormancy in this species depends on the position of seeds inside the hygrochastic capsules. Seeds in the upper part of capsules germinated immediately after dispersal while those located on the bottom of capsules retained dormancy for several years (Gutterman, 1980). Future analyses should verify, whether similar position effects are existent in *Lampranthus* seeds and ovules. If so, ovules should be selected carefully in coming embryo rescue approaches. Furthermore, seed germination in *M. nodiflorum* was found to follow a seasonal rhythm (uniform germination during the winter months), which was retained even under laboratory conditions and therefore was not influenced by environmental factors (Gutterman, 1980). More recent investigations revealed that the germination pattern of *M. nodiflorum* does not change over time, even after storage of seeds for 32 years. However, the physiological backgrounds of seed dormancy and germination in this species are still unknown (Gutterman and Gendler, 2005) and similar reports are missing in the Ruschioideae subfamily. Due to the high impact of temperature on growth and flowering of many middy flowers (see above), it might as well be considered as crucial factor for ovule and seed germination in *Lampranthus*. However, in a temperature range from 8 °C to 24 °C, Esch (2016) detected no significant differences in *Lampranthus* seed germination. Decreasing germination rates were observed at 28 °C and complete inhibition of seed germination at 3 °C and 33 °C.

6.4 Occurrence of unreduced male gametes

Although polyploid specimens were occasionally detected within boundaries of Ruschioideae species (e.g. Albers and Haas, 1978; Hartmann, 1986), up to now no studies furnishing proof for the occurrence of unreduced gametes in the Aizoaceae exist. However, in line with the observations made in the investigated *Lampranthus* and *Delosperma* genotypes, Suehs et al. (2006) detected macro pollen in different species of *Carpobrotus* (Ruschioideae). They assumed that the observed macro pollen were diplogametes because meiotic abnormalities (microspore diad and triad formations) had been previously detected in the same species by Diadema (2002).

In many studies, the presence and frequency of unreduced pollen grains were determined by morphological screenings (Bretagnolle and Thompson, 1995), as their diameters are typically 30 - 40 % larger than those of reduced pollen grains (Ramsey and Schemske, 1998). Thus, the positive correlation found between nuclear DNA contents and cell volumes in somatic tissues (e.g. Jovtchev, 2006) seems to be valid also for pollen. However, efforts made to correlate the varying diameters of midday flower pollen grains with different ploidy levels failed, because the pollen sizes followed an almost normal distribution (Chapter 4). Bell (1959) detected for the first time that pollen diameters in *Solanum lycopersicum*, *Petunia sp.* and *Portulaca grandiflora* were highly influenced by mineral nutrition. Consequently, he emphasized the danger of using pollen size as an indicator of polyploidy or as a taxonomic characteristic. In the cactus *Pachycereus weberi*, which belongs to the same taxonomic order (Caryophyllales) as midday flowers, Aguilar-García et al. (2012) discovered extremely varying pollen grain diameters (5 - 950 µm). Jansen and Den Nijs (1993) described overlapping of haploid and diploid pollen grain diameters in *Lolium perenne*. They developed a statistical model for the determination of 2n pollen occurrence, which relied on the assumption that

the size of haploid pollen grains is normally distributed, and that the size of diploid pollen grains is normally distributed with the same variance. However, the authors have not verified their results by using alternative screening methods.

As measurements of pollen size gave no information about the presence of 2n gametes in the investigated genotypes, further methods that allow direct determination of ploidy levels in intact pollen grains are desirable. Recently, De Storme et al. (2013) presented a volume-based screening approach, in which unreduced pollen grains of *Arabidopsis thaliana* and *Boechera polyantha* were identified using a Coulter counter.

The analysis of microsporogenesis (Chapter 5) mainly focused on the occurrence of increased bivalent numbers during diakinesis, which would have been an indicator for pre-meiotic endomitosis, and on the presence of microspore diads, triads and monads, which would have indicated meiotic division restitution. None of these aberrations were observed in the investigated midday flowers. In other studies, analyses of microsporogenesis were successfully performed in order to describe the underlying mechanisms of 2n gamete formation, e.g. in *Rosa* (Pécrix et al., 2011; Crespel et al., 2006), *Begonia* (Dewitte et al., 2009) and *Arabidopsis* (De Storme and Geelen, 2011). Generally, analyses of microsporogenesis are laborious and time-consuming. Moreover, De Storme and Geelen (2013a) point to the fact that the detection of pre-meiotic abnormalities is not a simple task. Nevertheless, as these microscopic investigations are most informative in comparison to other methods, they should also be included in future experiments.

Actually, flow cytometry was considered as a much more efficient technique to determine the rates of 2n gamete formation (Bretagnolle and Thompson, 1995). However, in line with recent findings of Hirano and Hoshino (2009) and Kron and Husband (2015) in other plants, paired midday flower sperm nuclei and intact male germ units (MGUs) caused the detection of fluorescence signals corresponding to DNA contents of 2C and 3C, and possibly masked diploid pollen nuclei. Kron and Husband (2015) presented a possibility for "doublet correction" in flow cytometric data, which is based on pulse analysis. Hereby, not only one-dimensional fluorescence intensities, but also fluorescence "heights", "widths", "areas" and their ratios are taken into account for nuclei identification. As pulse analysis is only well applicable if the nuclei of interest are spherical and homogeneous in shape (Kron and Husband 2015), it is probably not appropriate for analysis of Aizoaceae pollen. In observations by fluorescence microscopy, vegetative nuclei were highly heterogeneous and sperm nuclei had an elliptical shape after extraction from pollen tubes. Future approaches should rather include the cleavage of MGU associations without damaging the nuclei themselves. Ge et al. (2011) disrupted *Arabidopsis* MGUs in pollen tubes by mild temperature elevation (29 - 31 °C) for one hour. In the investigated midday flowers, however, this treatment resulted in complete decomposition of nuclei (not shown).

A further method to verify the presence of unreduced gametes (but not the rates of their formation) are interploidy crosses and subsequent progeny analysis (Bretagnolle and Thompson, 1995). After polyploidizations *in vitro*, this strategy could be also applied to the compatible *Delosperma* genotypes.

6.5 Small genome sizes and the physiological significance of endoreduplication in the Aizoaceae

In respect of genome sizes, Soltis et al. (2003) classified the Aizoaceae as `very small´ (< 1.4 pg/2C), although at this time nuclear DNA contents were only known in one species (*M. crystallinum*; 0.9 pg/2C; De Rocher et al., 1990; Bennett and Leitch, 1995). The reason for the scarcity of genome

size data for midday flowers is perhaps due to the fact that flow cytometric measurements are difficult in endoreduplicative tissues. Endocycling cells might interfere with the internal calibration standard or overlap its fluorescence signals (see also Braun, 2012). In the approach described in Chapter 5, this problem was circumvented by measuring pollen nuclei instead of endocycling somatic nuclei. Alternatively, somatic nuclei could be measured together with standards that potentially possess lower DNA contents than the investigated plants (e.g. *Arabidopsis*). The analysis revealed nuclear DNA contents of 1.8 - 3.68 pg/2C in *Delosperma* and of 1.6 – 2.36 pg/2C in *Lampranthus*, which definitely fall at the low end of the range of known plant genome sizes (e.g. Greilhuber et al., 2006; Pellicer et al., 2010). Unfortunately, the taxonomic background of most investigated genotypes was not known in detail, but the methodology presented in this thesis might allow comprehensive insights into genome size variations within the Aizoaceae in future surveys. According to Ohri (1998), comparison of nuclear C-values provides a possibility to elucidate evolutionary relationships within narrow taxonomic groups. In addition, flow cytometric analysis of pollen nuclei could be used to verify the hybrid state of seedlings that may be obtained in coming crossbreeding experiments. However, this technique can only be applied if the genome sizes of the parental species are sufficiently different (Winkelmann et al., 2010) and if the potential hybrid is assumed to possess an intermediate genome size. The small genome size that was determined in the intergeneric *Delosperma* x *Lampranthus* hybrid and the low genetic similarity to the paternal genotype (Chapter 3) suggest that this might not necessarily be the case in midday flower breeding.

Due to endoreduplication, the investigated plants show plasticity in nuclear DNA contents with at least five different C-levels. In line with the findings of De Rocher et al. (1990), the flow cytometric analyses decribed in Chapters 4 and 5 furthermore revealed that endoreduplication is differently expressed in plant organs. Presumably, endoreduplication fulfills various functions in midday flowers. Increased cell expansion allows faster and more energy-efficient growth (e.g. in cotyledons, primary roots and petaloid staminodes) because in contrast to cell division, processes such as spindle formation, chromosome condensation and decondensation, and reconstruction of nuclear envelopes are not needed (Barow, 2006). Due to the positive correlation between DNA content and cell size (e.g. Jovtchev, 2006), endoreduplication presumably contributes to the development of water-storing tissues (e.g. fully developed leaves) in the investigated genotypes. As the genome sizes of midday flowers appear to be generally small, endoreduplication might have been an important prerequisite for the evolution of leaf succulence in the Aizoaceae and therefore can be considered as an adaption to arid environments (see also De Rocher et al., 1990; Del Angel et al., 2006). Certainly, the provision of high DNA amounts to support high metabolic demands in specialized cells (Barow, 2006) is another important function of endoreduplication in midday flowers. However, the performed experiments were inadequate to clarify cell metabolism issues.

Whether the proportions of non-endoreduplicated cells in plant organs can be used as selection criterion for explants in tissue culture approaches that target organogenesis, should be tested in coming experiments. In addition, microscopic analyses could clarify whether organogenesis is exclusively restricted to non-endoreduplicated cells or if deduplications (as described by Galbraith et al., 1981; Valente et al., 1998) as well allow endocyling midday flower cells to return to mitosis.

6.6 Conformance with the thesis objectives

This paragraph reverts to the objectives of this thesis described in Chapter 1.3.5 and explains to which extent the objectives were achieved by the described investigations.

- Determination of flower-inducing factors and of how these could be controlled in a greenhouse environment

The analyses showed that photoperiod was not a crucial factor to flower induction in *Cephalophyllum*, *Lampranthus* and *Delosperma*, but temperature was found to play a prominent role (Chapter 2). Although further analyses at different temperature ranges and inclusion of other genotypes are needed, the results of this thesis indicate that temperature control is essential for midday flower breeding and production.

- Localization and overcoming of hybridization barriers after intra- and intergeneric cross-pollinations

The investigations revealed that hybridization barriers predominantly occurred after fertilization (Chapter 3). In most cases, hybridizations failed due to abnormal early embryogenesis (most likely resulting in embryo abortion), albinism and hybrid breakdown. As shown by the confirmation of hybridity in some plants that were derived from cross-combinations, the reproductive isolation mechanisms within and between the investigated genera were leaky. On the one hand, the relatively weak expression of hybridization barriers might have contributed to the hyperdiversity of the Aizoaceae and on the other hand offers incentives to test further wide hybridizations of midday flowers in breeding programs.

- Characterization of self-incompatibility mechanisms

All investigated genotypes were found to be self-incompatible (Chapter 3). While pollen tube growth in *Delosperma* was inhibited on the stigma surface after self-pollinations, self-incompatibility in *Lampranthus* most likely relied on fertilization failures or postzygotic barriers. Thus, in breeding approaches that are aimed at generating hybrid progeny, flower emasculations are unnecessary. However, self-incompatibility will hinder the generation of inbred lines in midday flower breeding.

- Identification of spontaneously formed unreduced male gametes and an evaluation of how these could be utilized for polyploidizations or overcoming of ploidy-related hybridization barriers

Although there were signs for the presence of unreduced pollen grains in the investigated genotypes, none of the applied methods could prove their existence. Nevertheless, the analyses provided valuable insights into pollen formation and cytology and allowed chromosome counts in one genotype. Furthermore, the obtained findings reinforced that flow cytometric screenings entail the risk of false positive results due to sperm doublet and MGU detection.

- Estimation of genome sizes by flow cytometric analyses

The genome sizes of several genotypes were successfully estimated by measuring isolated pollen nuclei together with internal calibration standards. This approach might also be useful for genome size estimations in other plants with polysomatic tissues, as it circumvents the problem of overlapping fluorescence signals. Generally, the obtained genome sizes can contribute to the prediction of cross-compatibility in future breeding approaches.

- Characterization of endoreduplication (endopolyploidy) as a prerequisite for *in vitro* polyploidizations

The analyses revealed remarkably high extents of endoreduplication in all investigated genotypes. However, the endoreduplication patterns appeared to be organ-specific and some organs were found to contain relatively low proportions of endocycling cells. These results provide the basis for future experiments, in which explants with different endoreduplication patterns will be used for shoot regeneration *in vitro*.

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Laß die Moleküle rasen,
was sie auch zusammenknobeln!
Laß das Tüfteln, laß das Hobeln,
heilig halte die Ekstasen.

(Christian Morgenstern, 1905)