The evolution and prevalence of reproductive assurance in the genus *Lachenalia*

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ABSTRACT

Since its proposal by Darwin (1876), the concept of reproductive assurance (RA) has been much discussed, modified and tested. It is hypothesized to occur under pollen- and/or mate-limitation, each of which can arise in a multitude of manners, and RA itself takes many forms. Here its evolution and prevalence in the genus Lachenalia (Asparagaceae) is investigated. The genus exhibits many of the characteristics suggested in the literature to be associated with reproductive assurance, including intraspecific polyploids, vegetative reproduction and self-compatibility. Of the 27 study species, 20 were found to be self-compatible and four were capable of autonomous self-pollination. Pollinator-dependent species were found to have more attractive floral display structures. The five species exhibiting intra-specific polyploidy were all self-compatible. Offset production, known to occur in many Lachenalia species, was thought to exhibit a negative association with self-compatibility. No significant association, however, was found. A deeper understanding of the patterns of reproductive assurance in the genus requires study of the mechanisms enforcing self-incompatibility, and of the ecological and physiological correlates of reproductive assurance, is needed.

INTRODUCTION

Outcrossing and its evolutionary benefits

Outcrossing, also known as cross-pollination, is often enforced by self-incompatibility, the main purpose of which is suggested to be the avoidance of inbreeding depression (Goldberg et al. 2010). Inbreeding depression occurs when offspring resulting from self-pollination exhibit lower survival rates and fertility than offspring resulting from cross-pollination. Two theories have been proposed: overdominance, and partial dominance. The former theory attributes inbreeding depression to a fitness advantage of heterozygotes over homozygotes; the latter, to the fixation of deleterious alleles in the offspring of closely-related parents (Charlesworth and Charlesworth 1987). The consequences of inbreeding depression depend on the mating system of the species concerned. Inbred individuals of a usually outbreeding species are likely to experience lowered fitness and reproductive success. However, self-fertilizing species may be able to purge deleterious alleles from the gene pool, so that inbreeding

depression due to partial dominance is relatively low (Charlesworth and Charlesworth 1987). Schemske and Lande (1985) showed that self-fertilization occurring at a rate of only 10% in a population could significantly reduce the level of inbreeding depression in that population.

The costs of outcrossing

Outcrossing requires a larger investment in the male sexual function than in the female sexual function (Cruden and Lyon 1985), specifically in the production of pollen (Cruden 2000). Much of a flower's pollen will be transported by pollinators to other species, lost en route or consumed by pollinators, so enough must survive these dangers to eventually fertilize an ovule (Cruden 2000). The energy invested in sexual functions and in floral display is wasted if no pollinators and/or no suitable mates are available. In such situations the individual will not only be unable to produce offspring but will have wasted resources that may have been used for other processes to ensure survival until the following breeding season.

Outcrossing presents unique problems for clonal species. In species in which clones do not disperse far and thus establish near the mother plant, patches (genets) of genetically-identical individuals (ramets) will occur throughout each population. A pollinator visiting an individual in the centre of such a patch is likely to proceed to another individual within the patch, rather than to one further away; thus the receiving individual is self- rather than cross-pollinated (Vallejo-Marin et al. 2010). Ramets on the periphery are more likely to receive outcross pollen as they are situated adjacent to ramets of a different genet (Handel 1985; Reusch 2001). The frequency of outcrossing in clonal species increases with the degree of dispersal the organs of vegetative reproduction and the resultant amount of intermingling of genets.

The evolution of self-compatibility

Self-incompatibility is enforced by genes which allow plants to recognise and reject their own pollen; and is maintained by different genetic mechanisms in different taxa, having arisen independently multiple times amongst the angiosperms. The most common genetic controller of pollen-recognition is the polymorphic S-locus, often accompanied by other genes (Takayama and Isogai 2005; Igic et al. 2008). Therefore, pollen-recognition is regulated by a number of genes, and so is difficult to develop from scratch after a transition to self-compatibility, hindering reversions (Marshall et al. 1994, Igic et al. 2006; both cited in Igic et al. 2008). Additionally, the shift to self-compatibility is generally accompanied by

morphological changes which enhance its efficiency and simultaneously reduce the efficiency of outcrossing, for example, a reduction in pollen-production (Lloyd 1992). The evolution of self-compatibility is usually seen as a one-way road; easier to evolve than to turn back from (Igic et al. 2008).

Self-compatibility as a means of reproductive assurance

Self-fertilization may offer a range of benefits, from increased ease of establishment of new populations (Baker 1955) to increased seed production (Darwin 1876; Fisher 1941 cited in Busch and Delph 2012), but it also reduces genetic diversity, viability and diversification (Stebbins 1957 cited in Goldberg et al. 2010; Busch and Delph 2012). Additionally, all its benefits are discounted by inbreeding depression (Busch and Delph 2012). Why, then, is self-fertilization sometimes selected for over outbreeding?

Automatic selection states that self-fertilization is favoured because a self-fertilizing parent's genes are present in both gametes, and therefore each allele is twice as likely as that of an outcrossing parent to be transmitted to its offspring (Eckert et al. 2006; Barrett 2010; Busch and Delph 2012). Provided that the increase in transmission has benefits that outweigh the reduction in fitness of the offspring through inbreeding depression, self-fertilization will be selected for (Busch and Delph 2012).

The second hypothesis relates to reproductive assurance, the ensuring of seed set when pollen is limiting, due to low pollinator- and/or mate-availability. Autonomous self-pollination might be selected for when pollinators are absent, as it would allow individuals to pass on their genes despite not being able to outcross (Busch and Delph 2012). Alternatively, geitonogamous self-pollination would allow an individual to fertilize its ovules with self-pollen transported between its own flowers by pollinators (Busch and Delph 2012) when mates are absent.

The polyploid factor

Mate-limitation not only occurs when conspecifics are separated by greater distances than pollinators are likely to travel. Indeed, populations may be dense, and yet individuals still experience mate-limitation, if they belong to a minority polyploid type. Mate-limitation occurs because mating of individuals of different ploidy levels rarely produces fit offspring; if a tetraploid (4n) individual mates with a diploid (2n), the resultant offspring are triploid (3n). The unpaired chromosomes in the triploid often prevent successful meiosis, resulting in sterility (Campbell and Reece 2002). However, tetraploids can reproduce through self-pollination. The two may in fact be intrinsically associated: Lewis 1949 (cited

in Marks 1966) observed that tetraploid plants did not recognise their diploid pollen as their own, and so accepted it for fertilization. Assuming that the movement of pollinators between individuals is random and that polyploid individuals are in the minority in a population, the pollen of polyploid individuals is more likely to be transported to diploid individuals than to other polyploids, and polyploids are more likely to receive pollen from diploid individuals than from other polyploid individuals. As the polyploids are unlikely to be maintained through outcrossing alone, they should experience selection for self-compatibility.

The morphological correlates of autonomous self-pollination

Species capable of autonomous self-pollination have been noted to bear relatively small, inconspicuous flowers (Cruden and Lyon 1985; Cruden 2000). They should experience less selection pressure to attract pollinators as they are not exclusively dependent on outcrossing for setting seed. Characters which are involved in creating floral displays that attract pollinators are scent, flower size, flower colour, nectar quantity, and inflorescence size. In species capable of autonomous self-pollination these characters are thought to experience less selection for attractiveness relative to those that are pollinator-dependent. The characteristic floral traits of autonomous self-pollinators are collectively termed the 'selfing syndrome' (Darwin 1876; Ornduff 1969; Richards 1986 in Sicard and Lenhard 2011). Such species have less scented, more dully coloured and less widely opening flowers than outbreeding sister taxa. They also tend to have smaller flowers (Goodwillie et al. 2010; Sicard and Lenhard 2011), and have fewer flowers open simultaneously (Goodwillie et al. 2010). Flower size and flower number are often considered key components of floral display (*mention some examples of studies); their reduction in self-pollinating species reflects diminished dependence on pollinators in these species. The theory of sex allocation assumes that sex allocation is dictated by limited resource availability and intrinsic trade-offs between allocation to the functions of each sex (Charlesworth and Charlesworth 1981; Charlesworth and Morgan 1991; Charnov 1982, West 2009; both cited in Sicard and Lenhard 2011). The reduction in pollen wastage afforded by intrafloral pollination has selected for lower pollen production, and accordingly higher ovule production (Sicard and Lenhard 2011).

The morphological correlates of geitonogamous self-pollination

The likelihood of a pollinator visiting multiple flowers per visit to a plant increases in accordance with the number of flowers borne by the plant. Only the first flower visited receives mostly outcross pollen; the pollen received by subsequent flowers on that individual will originate primarily from its own

flowers. Since most flowers will receive more self than outcross pollen, geitonogamous self-pollination should be selected for in species which produce many simultaneously-receptive flowers.

Vegetative reproduction as a reproductive assurance mechanism, and its relationship to self-compatibility

Geophytes are plants which possess an underground storage organ from which their leaves, stems and flowers develop. The underground structure stores nutrients and water on which the plant survives during regular periods of dormancy and which fuel maintenance, growth and reproductive functions when environmental conditions limit photosynthetic productivity and water availability (Dafni et al. 1981). Underground storage organs must reach a certain mass, that is, they must accumulate a certain amount of nutrient and water reserves, in order for the plant to flower. This 'juvenile period' differs in length according to species (Dafni et al. 1981). Some geophytic species reproduce clonally through the production of offsets, also known as bulblets or bulbils, by the main bulb. Vegetative reproduction is also known as clonal reproduction or asexual reproduction.

The reproductive assurance hypothesis as it was originally formulated refers only to the ability of plants to set seed through autonomous pollination in the absence of pollinators (Baker 1955; Schoen et al. 1996 cited in Vallejo-Marin et al. 2010). The concept has since been expanded to include all forms of reproduction other than seed-setting through outcrossing (Baker 1955; Eckert et al. 2006; Vallejo-Marin and O'Brien 2007 cited in Vallejo-Marin et al. 2010), i.e. seed production through autonomous self-pollination in the absence of pollinators, geitonogamous self-pollination when pollinators are present but suitable mates absent; and clonal reproduction (also known as vegetative reproduction, as seeds are not produced), in which the parent plant produces vegetative units which grow into independent individuals (Vallejo-Marin et al. 2010).

Assuming that vegetative reproduction and self-compatibility each independently provide adequate reproductive assurance, taxa that have evolved one should not also experience selection for the other. If, however, clones do not receive enough outcross-pollen, then self-compatibility may be selected for (Vallejo-Marin et al. 2010). Dividing available resources between two processes which fulfil the same purpose would violate the theory of resource allocation, according to which resources allocated to one avenue of reproductive assurance would allow correspondingly fewer resources to be allocated to the other. Were both to be invested in, the success of each would be diminished. Quantifying the resource

consumption of clonal growth units is complicated by the varying times for which they are dependent on the parent plant in different taxa, and by their ability to contribute to their own growth (Ogden 1974 in Bazzaz et al. 2000). Thus the extent to which vegetative reproduction utilizes resources which would have otherwise been available for sexual reproduction may be unclear.

Lachenalia as a study system for exploring the evolution of autogamous self-pollination

Lachenalia Jacq. f. ex. Murray is a genus of approximately 115 (Duncan 1996, Duncan 1998; both in Duncan et al. 2005) geophytic, bulb-producing species endemic to South Africa and Namibia (Kleynhans and Hancke 2002; Duncan et al. 2005). Species are deciduous and winter-growing, and most diverse in regions of winter-rainfall, exhibiting the highest diversity in the Succulent Karoo biome (Duncan 2005; Duncan et al. 2005) Species exhibit diverse morphologies and ranges in ploidy level (including intraspecific polyploidy) and basic chromosome number (find refs for polyploidy; Kleynhans and Hancke 2002; Duncan et al. 2005), with chromosome counts available for only 55 species (Moffett 1936, de Wet 1957, Riley 1962, Ornduff and Watters 1978, Nordenstam 1982, Crosby 1986, Hancke and Liebenberg 1990, Hancke 1991, Duncan 1993, Duncan 1996, Johnson and Brandham 1997, Hamatani et al. 1998, Kleynhans and Spies 1999, Spies et al. 2000, Spies et al. 2002, van Rooyen et al. 2002; all in Duncan 2005). Additionally, some species have been noted to set seed in the absence of pollinators (Duncan, pers. comm.) As a result, the delimitations, and taxonomic relationships, between some species have undergone multiple revisions (Crosby 1986 in Kleynhans and Hancke 2002; Duncan 1992 in Duncan et al. 2005).

Vegetative reproduction through offset production (formation of daughter bulbs in a ring around the mother bulb) was observed by Duncan (2005) to occur in 79 species. An additional three species reproduce vegetatively through the production of bulbil-bearing stolons by the bulbs. These organs develop towards the end of the growing season. Floral display varies from a few flowers presented at ground-level to >40 flowers borne on tall, sturdy inflorescences (Duncan 2005).

The approach followed in this study

This study combined hand-controlled pollination experiments with morphological assessments of floral and offset attributes and phylogenetic reconstructions to examine the prevalence and evolution of reproductive assurance in the genus *Lachenalia*. Four hypotheses were tested:

a) Autogamous self-pollination is derived, its evolution being dependent on: (i) the prior evolution of self-compatibility, and (ii) associated with a reduction in the spatial separation of male and female parts within the flower.

b) Autogamous self-pollination is associated with a reduction in the numbers of flowers produced, and the size of the reproductive display (measured in terms of inflorescence height and floral diameter).

c) Autonomous self-pollination is associated with a peak flowering time that is furthest from the peak spring flowering period (when pollinators are most abundant). Species capable of autonomous self-pollination lack alternative reproductive assurance mechanisms (e.g. vegetative reproduction).

d) Autonomous self-pollination is associated with intraspecific polyploidy, resulting in a correlation between these traits.

METHODS

Specimens

The specimens used in this study were selected from the Lachenalia collection under care of Graham Duncan at Kirstenbosch. As this study was begun in February and needed to be completed by October, species were chosen by the timing of their flowering and seed-production. However, not all suitable species were present in the collection, and some that were did not flower. The species on which pollination experiments were conducted are listed in Table 1.

The specimens in this collection are potted according to species and collection trip, with a stake in each pot noting species, collector, accession number and date and location of collection. One pot of each species was selected, the contents poured out, and suitable bulbs selected for use in this study. The larger bulbs of each pot were selected, as, being older individuals, they were more likely to flower in the forthcoming growth season. When conjoined bulbs were present, only one was selected, if there were enough bulbs in the pot to allow this without falling below 5 replicates, as conjoined bulbs are offsets produced by a single mother bulb, and so are genetically identical. Bulbs were potted in new pots in a 1:1 river sand:compost mix, with old terracotta pot shards or gravel pieces sprinkled first sprinkled into the pot to provide adequate drainage yet prevent soil loss through drainage holes. Bulbs were spaced evenly and set deep enough so that their necks were just below the soil surface. Each pot was identified

with a stake noting at least species and accession number; comprehensive details of each collection and the date of repotting were recorded in a study notebook.

The pots were placed in the greenhouse containing the main Lachenalia collection. Netting was sewn over a domed metal frame and the structure was used to cover the study specimens to further exclude pollinators; insects and sunbirds are known to occasionally enter the greenhouse through holes in its netting.

Pollination experiments

The plants were watered regularly once leaf shoots began developing. A non-toxic, xylene-free Penflex permanent marker was used to number individuals of each species on their leaves. One flower of each plant was marked for each treatment (autonomous self-pollination, geitonomous self-pollination and outcrossing) with a length of string tied loosely around the pedicel. A single treatment or a randomly-selected pair of treatments was applied to individuals bearing fewer than three flowers. A length of string, colour-coded to indicate treatment type, was tied loosely around the peduncle of each treated flower. Implements were sterilized before and after each treatment with 70% ethanol. Flowers selected for autonomous self-pollination were left untouched after marking with string.

Table 1. Study species, their flowering periods and known pollinators, and habitats. Flowering periods, habitats and pollinators are sourced from Duncan (2005). Honey bees referred to are *Apis mellifera*.

Species	Species code	Flowering period	Habitat	Pollinator(s)
L. aloides	ALO	Jun-Jul	Renosterveld	Sunbirds
L. barkeriana	BAR	Apr-May	Succulent Karoo	?
L. bolusii	BOL	Jul-Aug	Succulent Karoo	Honey bees
L. buchubergensis	BUC	May-Jul	desert, Succulent Karoo	?
L. calcicola	CAL	May	Fynbos	Honey bees
L. congesta	CON	Jun-Jul	Renosterveld	Honey bees
L. corymbosa	COR	Apr-Jun	Renosterveld	Honey bees
L. ensifolia	ENS	Apr-Jun	Succulent Karoo, Nama Karoo, Fynbos, Renosterveld	Honey bees; butterflies
L. framesii	FRA	Jul-Aug	Succulent Karoo	Honey bees
L. inconspicua	INC	Jul-Aug	Succulent Karoo	Honey bees
L. karooportensis	KAR	Jul-Aug	Succulent Karoo, Fynbos	Honey bees
L. klinghardtiana	KLI	Jun-Jul	desert, Succulent Karoo	Honey bees

L. orchioides	ORC	Aug-Oct	Fynbos	Honey bees
L. marginata	MARG	Jul-Aug	Succulent Karoo, Fynbos	?
L. martinae	MART	Jul	Succulent Karoo	?
L. maximiliani	MAX	Jul-Aug	Succulent Karoo	Honey bees
L. minima	MIN	May-Jul	Renosterveld	Honey bees
L. paucifolia	PAU	Apr-Jun	strandveld	Honey bees
L. punctata	PUN	Mar-Jul	strandveld, Fynbos	Sunbirds
L. pusilla	PUS	Apr-Jun	Renosterveld, strandveld	?
L. pygmaea	PYG	Apr-Jun	Succulent Karoo	Honey bees; butterflies
L. quadricolor	QUA	Jun-Jul	strandveld	Sunbirds
L. reflexa	REF	Jun-Aug	Renosterveld	?
L. schelpei	SCH	Jul-Aug	Succulent Karoo	?
L. splendida	SPL	Jul-Aug	Succulent Karoo	Honey bees
L. valeriae	VAL	Jun-Jul	Succulent Karoo	Honey bees
L. viridiflora	VIR	May-Jul	strandveld	Sunbirds

The stigmas of flowers selected for geitonomous self-pollination were dusted heavily with an anther plucked with fine forceps from another flower of the same individual. The anther was held with the forceps and brushed against the receiving flower's stigma. Anthers from flowers marked for autonomous self-pollination were not used to avoid accidental pollination of these flowers and disruption of any structural mechanisms of autonomous self-pollination. Pollen from at least three conspecific individuals was collected, mixed with a fine brush and applied with that brush to the stigmas of flowers marked for outcrossing.

Flowers that were hand-pollinated (i.e. those marked for geitonomous self-pollination and outcrossing) were pollinated only once all anthers had dehisced. Treatments were often applied to each species over a number of days as individuals tend to flower in asynchrony and flowers within inflorescences, too, may be fully open and dehisced at different times (inflorescences in a number of species mature from the bottom up, so that flowers at the base may be withering while those at the top may just be opening).

<u>Collection of seed</u>

Each treated flower was monitored for development of a seed capsule, which was collected once ripe but before dehiscence. One species overlooked for treatment (*L. barkeriana*) was also examined for ovary swelling, since this species has been reported (Duncan 2005) to be capable of autonomous self-pollination. Capsules were stored with granules of silica desiccant until seeds were counted.

Quantification of floral display and style-anther separation

Inflorescence height was measured using digital callipers and flower number per inflorescence was recorded. Up to five flowers of each species were collected, each from a different individual. In most instances, flowers were collected from live specimens, but herbarium material was used in some instances. The fresh flowers were preserved in 70% ethanol until dissection. Dissections were performed with a Zeiss Stemi-2000-C microscope fitted with an eyepiece graticule. Peduncle length, flower height, corolla diameter, style height and anther height were measured. Mean style-anther separation was calculated for each species.

Quantification of self-compatibility and autonomous self-pollination

Self-compatibility was quantified at the species level with the index of self-incompatibility (ISI [Lloyd 1965; Bawa 1974, both cited in Robertson et al. 2011]):

$$= 1 - \frac{\text{mean proportion of geitonogamous self-pollination trials that resulted in seed set}}{\text{mean proportion of cross-pollination trials that resulted in seed set}}$$

Thus an entirely self-incompatible species will have an ISI = 1

Autonomous self-pollination, also at the species level, was quantified by species' pollinator-dependence (PD):

$$= \frac{\text{Mean number of seeds per flower from autonomous self-pollination trials}}{\text{Mean number of seeds per flower from cross-pollination trials}}$$

Thus an entirely pollinator-independent species will have a PD = 0

Autonomous-self pollination may produce no seed, some seed, or as much seed as cross-pollination. A species that produces equivalent amounts of seed through both pollination types receives the most reproductive assurance from selfing; such species are 'pollinator-independent'.

The measure of floral display employed by Goodwillie et al. (2010) was calculated at the species level thus:

$$floral\ display = \log_e (mean\ corolla\ diameter * mean\ number\ of\ flowers\ per\ inflorescence)$$

Phylogenetic analysis

Inference of phylogenetic relationships in *Lachenalia* was based on a combination of internal transcribed spacer (ITS, White et al. 1990) DNA sequence data and and morphological character data. A total of 25 *Lachenalia* species was included, with *Massonia depressa* included as an outgroup.

For ITS, sequences of nine species were available from GenBank, having been previously generated by Hamatani et al. (2008): *Massonia depressa* (AB305007)*, L. aloides* (AB304972)*, L. congesta* (AB625560)*, L. ensifolia* (AB305010) *L. orchioides* (AB304992)*, L. pusilla* (AB304996)*, L. reflexa* (AB439275)*, L. splendida* (AB305002), *L. viridiflora* (AB305006). Although DNAs were extracted from leaf material of all remaining species, and sampled using PCR, only a further four species yielded usable sequences: in some instances PCR failed to yield clear amplification products, while in others PCR yielded multiple products and unreadable sequences, possibly indicating the existence of paralogues – sequences that are ancestral genes that have undergone duplication (Lockton and Gaut 2005).

DNA was extracted according to the modified CTAB procedure described by Gawel and Jarret (1991). For each sample 700 µl of 2x CTAB extraction buffer was mixed with 1 µl of mercaptoethanol. The total volume was poured into a Nunc-type tube, mixed and placed into a water bath pre-heated to 65°C. Fresh leaves were pulverized with a small amount of sterile sand and liquid nitrogen using a mortar and pestle. Each sample of pulverized tissue was transferred to a 2 ml tube and 700 µl of the warmed CTAB buffer mix added. Each was hand-mixed and then transferred to the water bath for no less than 60 minutes. After incubation in the water bath, 600 µl of chloroform:isoamyl alcohol (24:1, v/v) was added to each tube. Each sample was mixed by inversion for five minutes, after which it was centrifuged at 12 000 rpm for five minutes. The supernatants were pipetted into 1.5 ml microfuge tubes and a volume of ice-cold isopropanol equal that of the supernatant was added. Each sample was briefly mixed by inversion before being stored at -20°C overnight. Thereafter, the isopropanol was discarded from each tube and 250 µl of 75% ethanol was added to each tube to wash the DNA pellets. The tubes were agitated gently to dislodge the DNA pellets and were centrifuged at 12 000 rpm for three minutes. The

ethanol was discarded and the tubes left to air dry. Once dry, 100 µl of sterile water was added to each tube in order to re-suspend the DNA pellets, and the samples were stored at -20°C.

The nucleotide sequences of the ITS 1, 5.8S rRNA and ITS 2 regions were amplified from the extracted DNA using the ITS 5 and ITS 4 primers of White et al. (1990). The PCR amplifications were conducted on an Applied Biosystems GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following cycle: an initial denaturation of 2 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 52°C, 2 minutes at 72°C, and a final extension of 7 minutes at 72°C. The PCR products were then stored at 4°C until being tested with electrophoresis. Electrophoresis was performed using 1% agarose gel containing ethidium bromide and successful PCR products sent to the DNA Sequencer Unit at Stellenbosch University for sequencing. Successful forward and reverse DNA sequences were then assembled and manually edited in ChromasPro version 1.7.5 (Technelysium Pty. Ltd.) before being aligned in BioEdit version 7.2.3 (Hall 2013), alongside the sequences of Hamatani et al. (2008). Alignment involved an initial alignment phasing using Clustal (Larkin et al. 2007) and a subsequent manual-alignment phase.

The use of a morphological data set in addition to ITS sequence data was necessitated by the lack of ITS sequences for some of the study species. For this purpose I made use of the 73 morphological characters coded by Duncan (2005) in his cladistic analysis of *Lachenalia* and *Polyxena*, retaining his character coding scheme but treating polymorphisms as polymorphisms rather than as an additional state.

In the absence of supported conflict between the trees obtained from the ITS and morphological data (not shown: the morphological tree lacked support), these data were analysed in combination using both parsimony and Bayesian inference. Parsimony signal was assessed in PAUP* version 4.0b10 (Swofford 2001) using 500 nonparametric bootstrap replicates (Felsenstein 1985), each employing a heuristic search with a simple addition sequence and TBR branch swapping. Bayesian inference was performed in Mr. Bayes version 3.1.2, implementing a metropolis-coupled Markov Chain Monte Carlo analysis (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). For this purpose, a mixed model was used, with a GTR + Γ model structure applied to the ITS sequence partition. Two independent runs were performed, each comprising four chains. Each chain was run for 10^6 generations and was sampled every 100^{th} generation. Convergence was assessed by comparing the likelihoods and trees

achieved by the two runs at stationarity. Since the two runs produced similar trees, only one of the two tree sets was used to generate a maximum clade credibility tree for use in subsequent analyses.

Ancestral character state reconstructions

Ancestral character states reconstructions of ISI, PD and AF were implemented in Mesquite version 2.75 (build 564) (Maddison and Maddison 2011) using the parsimony criterion. Each character was coded as categorical with two states, and states were assumed to be unordered. Characters were not treated as continuous because the lack of knowledge of branch lengths meant that the timing of state shifts could not be calculated. Treating character states as discrete better highlights major evolutionary shifts from one state to another. The categorical character states of species having continuous character states =±0.5 were coded as unknown. Although geitonogamous self-pollination and cross-pollination experiments were not performed on *L. barkeriana* and *L. calcicola*, these species' ISI values were coded as 0 because both produced seed in all autonomous self-pollination trials. Their PD values were coded as 0 despite the fact that seed numbers produced as a result of autonomous self-pollination could not be compared to those produced through outcrossing. Both species produced high numbers of seed per flower relative to the other species (19.50 and 19.67, respectively; see Table 2) through autonomous self-pollination. I therefore thought it unlikely that outcrossing would produce such large amounts of seed that their PD values would be greater than 0.5.

Phylogenetically independent contrasts

Phylogenetically independent contrasts (PICs) for floral display, offset production, stigma-anther separation, polyploidy, ISI and PD were calculated using the packages APE (Paradis et al. 2004) and picante (Kembel et al. 2010) in R. As it was not possible to calculate branch lengths, all branches were set to a length of one unit for the PIC analyses. The relationship between each contrast was analyzed using the linear model function in R. Linear models were forced through the origin; first suggested by Garland et al. (1992; in Legendre and Desdevises 2009), this has become standard practice for comparisons of independent contrasts (Legendre and Desdevises 2009; Goodwillie et al. 2010; Paradis et al. 2013). The literature was examined for records of species' base chromosome (n) and diploid (2n) numbers, whole-species polyploidy and intraspecific polyploidy.

RESULTS

Cladogram support

The consensus tree obtained by parsimony analysis exhibited two instances of polytomy and was poorly supported (see Figure 1 for bootstrap percentage values); the cladogram obtained by Bayesian analysis lacked polytomies and was somewhat better supported, although very strong support (PP=1.00) is only present for some of the terminal branches. The most basal divide occurs between the *L. paucifolia* clade and the remaining species. *Lachenalia congesta* appears to be sister to a more inclusive clade (PP=0.84; BS=93%) which contains the remaining species. The monophylly of this clade is poorly supported (PP=0.67; BS<50%). Within this clade lie two monophyletic divisions, both weakly supported by the parsimony analysis: the *L. barkeriana* group (PP=0.88; BS<50%) and the *L. minima* group (PP=0.80; BS<50%). The loss of vegetative reproduction, evolution of pollinator-independence and switch to self-incompatibility are homoplasic. Two reversions to self-compatibility also occurred.

Pollination experiments

The results of the pollination experiments (Table 2) indicate a range in ability amongst the study species to set seed through autonomous and/or geitonogamous self-pollination; four species set seed in every trial in which flowers were allowed to autonomously self-pollinate, and only three species exhibited complete reliance on outcrossing for setting seed.

Polyploidy

Pollinator-independence arose independently in two lineages (Tree 1); in the ancestor of *L. barkeriana* and *L. pusilla* and in *L. reflexa*. None of these are known to be polyploid species or to exhibit intraspecific polyploidy. Of those species which are known to contain intraspecific polyploids, all are self-compatible but none pollinator-independent. *Lachenalia ensifolia*, which Spies (2004) suggested may have a chromosome number of either n=6 or n=13, may thus be polyploid, and is self-incompatible.

Evolution of breeding system traits and stigma-anther separation

Autonomous self-pollination (Tree 1) appears to be derived, evolving in both cases in lineages which had previously evolved self-compatibility (Tree 1). Self-compatibility is ancestral and has been retained in most species, with self-incompatibility being derived in *L. ensifolia*, *L. minima* and the *L. inconspicua* clade. Two species within this clade appear to have reverted to self-compatibility.

Stigma-anther separation and breeding system traits appear to have evolved quite independently. Although autonomous self-pollination tends to occur in species with low or intermediate stigma-anther separation, low stigma-anther separation also occurs in most of the self-incompatible species, others exhibiting intermediate and high stigma-anther separation (Figure 1). The disconnect between breeding system traits and stigma-anther separation is further emphasized by the lack of correlation between the two (Figure 2), whether comparing TIP values (ISI: R^2 = -0.012; df = 25; p = 0.41, PD: R^2 = 0.0021; df = 25; p = 0.31) or PIC values (PD: R^2 = 0.034; df = 22; p = 0.32). Only ISI scores (Figure 2a) exhibit a significant, albeit weak, correlation to stigma-anther separation, and only when PICs are compared (R^2 = 0.31; df = 22; p < 0.005).

Associations between floral display and self-compatibility

Species' ISI scores (Figure 3a-c; Figure 4a) lacked significant correlations with any of the measures of display examined, while PD scores exhibited significant correlations with a number of measures of display. PD scores (Figure 3d-f) were positively correlated with mean flower number per inflorescence when TIP values were evaluated (R^2 = 0.17; df = 25; p < 0.05) but not when phylogeny was taken into account (R^2 = 0.12; df = 22; p = 0.13). PD scores were also correlated with mean inflorescence heights (Figure 4b; TIP values: R^2 = 0.29; df = 25; p < 0.005; PIC comparisons: PICs: R^2 = 0.19; df = 22; p < 0.05) and with $\log_e$(mean corolla diameter*mean number of flowers per inflorescence) (TIP values: R^2 = 0.41; df = 23; p < 0.0005; PIC comparisons: R^2 = 0.47; df = 21; p < 0.001).

Vegetative reproduction

The phylogenetic analysis (Figure 1) indicates that the presence of vegetative reproduction is the ancestral state for the species studied. This state exhibits a near-significant (Fisher Exact Test; p = 0.071) tendency to be lost in self-incompatible lineages, contrary to the expectation that loss would occur most often in self-compatible ones. Vegetative reproduction has, however, also been lost in the common ancestor of *L. barkeriana* and *L. pusilla*. Comparisons of phylogenetically independent contrast values show that the mean number of offsets produced per bulb of each species bears no correlation to those species' ISI (Figure 5a) and PD (Figure 5b) scores (ISI: R^2 = 0.011; df = 18; p = 0.69; PD: R^2 = 0.0068; df = 18; p = 0.72). Many species noted in Duncan (2005) as capable of vegetative reproduction did not produce offsets in this study. Of those that did, offsets were only produced by a subset of individuals; mean offset number per individual was calculated for this subset only.

Flowering time

While the majority of the autonomous species flower before the end of May (Figure 6), most of the self-incompatible species flower from June or July into August, with almost half flowering around the end of July/beginning of August period alone. The pollinators of many species are unknown; the one autonomous species for which information is available is bee-pollinated. Many of the pollinator-dependent species are also bee-pollinated and a couple are pollinated by both bees and butterflies. Bee-pollinated species flower throughout the winter months.

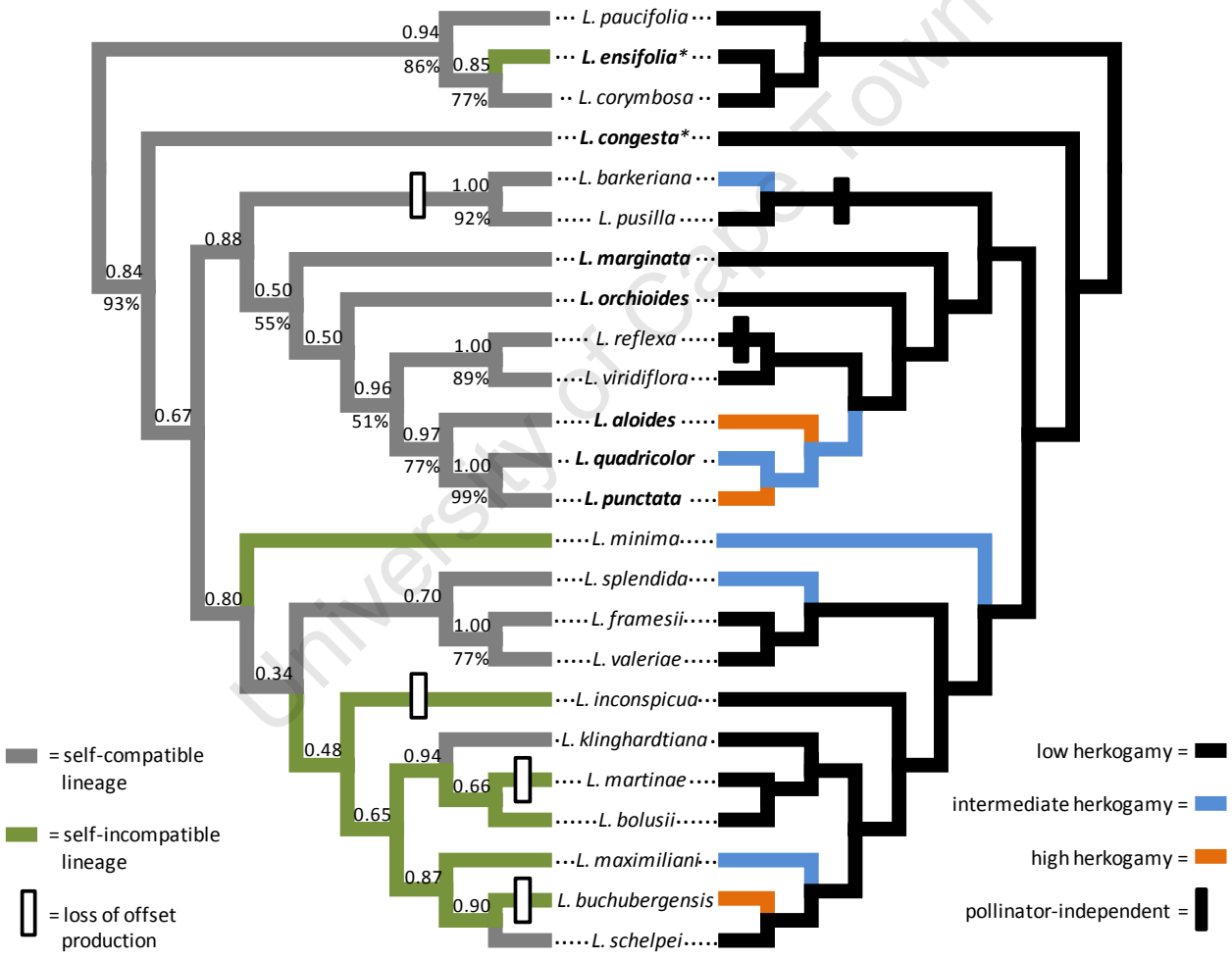

Figure 1. Cladogram of *Lachenalia species* constructed using a mixed-model Bayesian approach. All branches with poster probability (PP) ≥ 0.50 are shown; support values are shown above branches. Bootstrap percentages ≥50% are displayed below branches resolved in both parsimony and Bayesian inferences. Names in **bold** indicate intraspecific polyploidy; * indicates uncertainty as to base chromosome number (n), species may be polyploid; † indicates unknown base chromosome number; ‡ indicates knowledge of neither 2n nor n values.

Table 2: Results of pollination experiments. Fruit set values are proportions. Fruit set and seeds.flower^{-1} are species means. ISI = index of self-incompatibility, PD = pollinator independence. - indicates no experiments performed.

	Autonomous self-pollination			Geitonogamous self-pollination			Cross-pollination				
Species	Fruit set	Seeds. flower^{-1}	Sample size	Fruit set	Seeds. flower^{-1}	Sample size	Fruit set	Seeds. flower^{-1}	Sample size	ISI	PD
ALO	0.00	0.00	11	0.20	0.50	10	0.50	8.40	10	0.60	1.00
BAR	1.00	19.50	4	-	-	0	-	-	0	0.00	?
BOL	0.00	0.00	9	0.11	0.11	9	0.60	5.70	10	0.81	1.00
BUC	0.00	0.00	2	0.00	0.00	2	1.00	10.50	2	1.00	1.00
CAL	1.00	19.67	3	-	-	0	-	-	0	0.00	?
CON	0.00	0.00	5	0.25	0.50	4	0.75	10.50	4	0.67	1.00
COR	0.25	0.75	8	1.00	9.63	8	1.00	11.67	6	0.00	0.94
ENS	0.17	0.17	6	0.17	0.17	6	0.80	2.00	5	0.79	0.92
FRA	0.00	0.00	8	0.38	1.63	8	0.71	16.86	7	0.48	1.00
INC	0.00	0.00	5	0.20	0.40	5	0.60	10.20	5	0.67	1.00
KAR	0.20	0.20	5	0.40	1.80	5	0.60	3.80	5	0.33	0.95
KLI	0.00	0.00	11	0.64	1.18	11	1.00	10.91	11	0.36	1.00
ORC	0.00	0.00	10	0.70	2.30	10	1.00	21.00	11	0.30	1.00
MARG	0.00	0.00	7	0.50	1.00	6	1.00	20.00	4	0.50	1.00
MART	0.00	0.00	5	0.20	0.20	5	0.67	3.33	3	0.70	1.00
MAX	0.00	0.00	12	0.20	0.20	10	0.55	2.91	11	0.63	1.00
MIN	0.00	0.00	8	0.00	0.00	9	0.67	9.00	9	1.00	1.00
PAU	0.00	0.00	6	0.67	5.33	6	0.50	2.25	4	0.00	1.00
PUN	0.00	0.00	7	0.86	11.00	7	0.88	23.50	8	0.02	1.00
PUS	1.00	10.20	6	1.00	10.00	3	1.00	10.00	4	0.00	0.00
PYG	0.29	0.43	7	0.00	0.00	4	0.50	0.50	4	1.00	0.14
QUA	0.14	0.14	7	0.56	1.33	9	0.29	0.29	7	0.00	0.50
REF	1.00	41.25	4	1.00	22.50	2	1.00	22.00	2	0.00	0.00
SCH	0.00	0.00	5	0.40	0.40	5	0.40	0.80	5	0.00	1.00
SPL	0.00	0.00	10	0.40	0.50	10	0.80	9.00	10	0.50	1.00
VAL	0.00	0.00	6	0.33	1.00	6	0.36	2.18	11	0.08	1.00
VIR	0.00	0.00	6	1.00	5.80	5	0.83	34.17	6	0.00	1.00

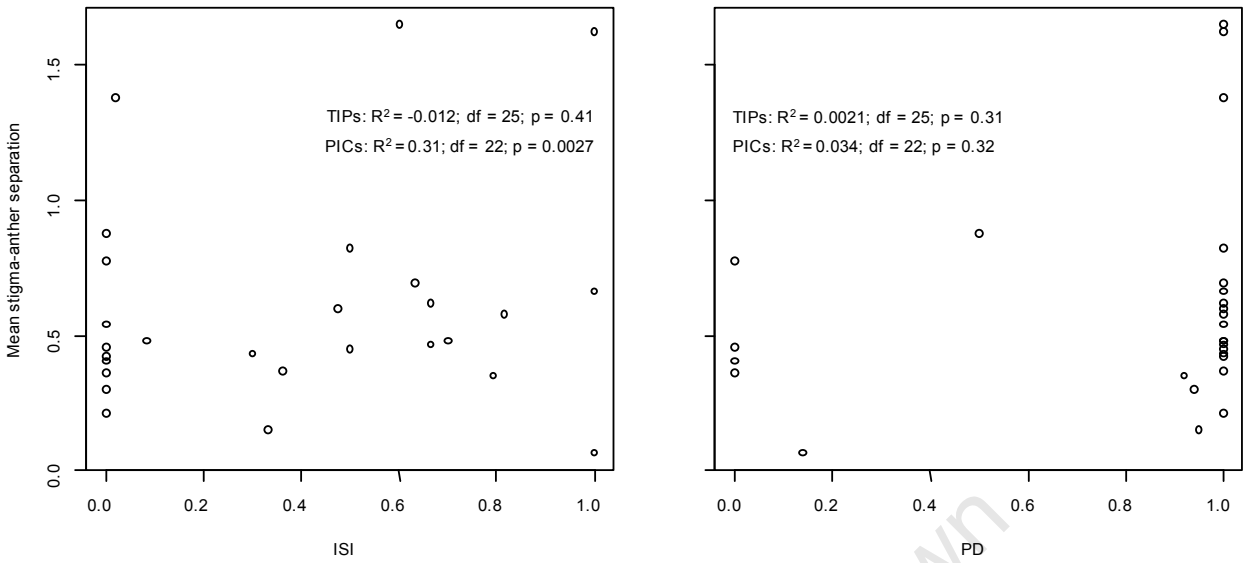

Figure 2. Associations between measures of self-compatibility and stigma-anther separation. ISI = index of self-incompatibility; PD = pollinator-dependence; stigma-anther separation values are absolute.

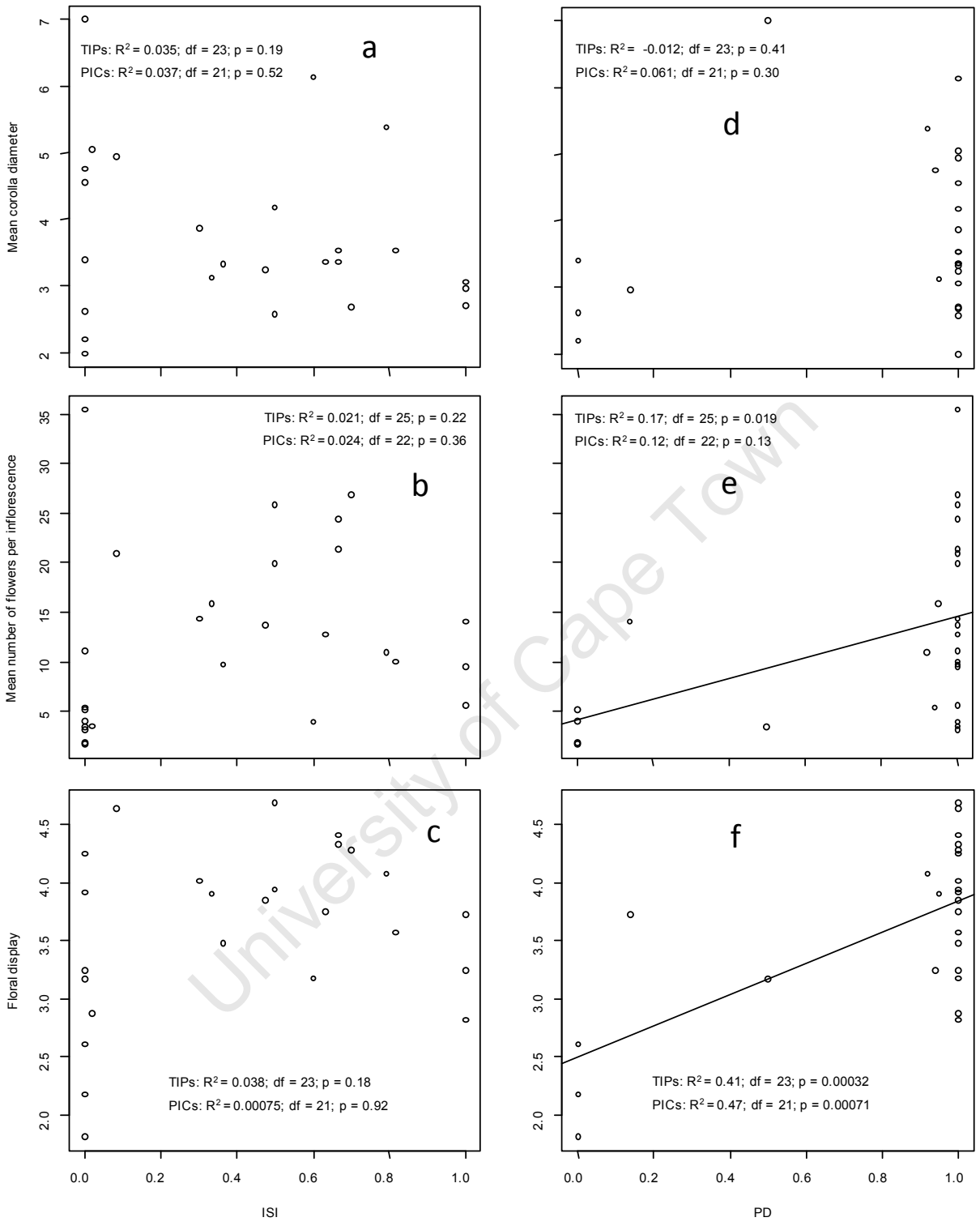

Figure 3. Associations between measures of self-compatibility, corolla diameter, flower number and floral display. ISI = Index of self-incompatibility; PD = pollinator-dependence. Floral display = $\log_e$ (mean corolla diameter*mean number of flowers per inflorescence).

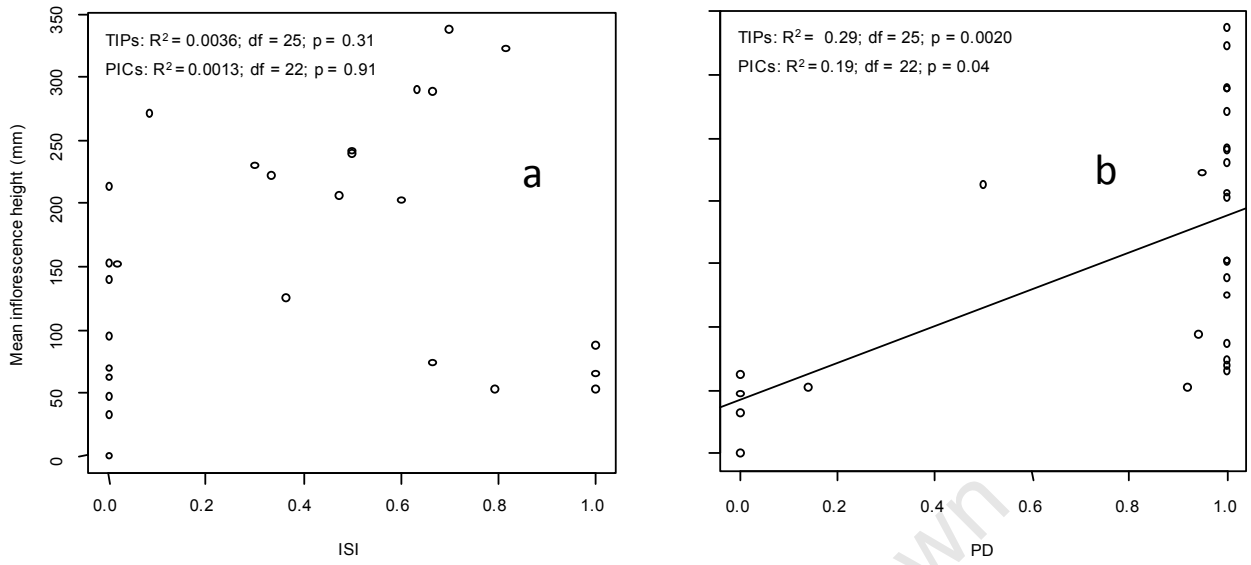

Figure 4. Associations between measures of self-compatibility and inflorescence height. ISI = index of self-incompatibility; PD = pollinator-dependence.

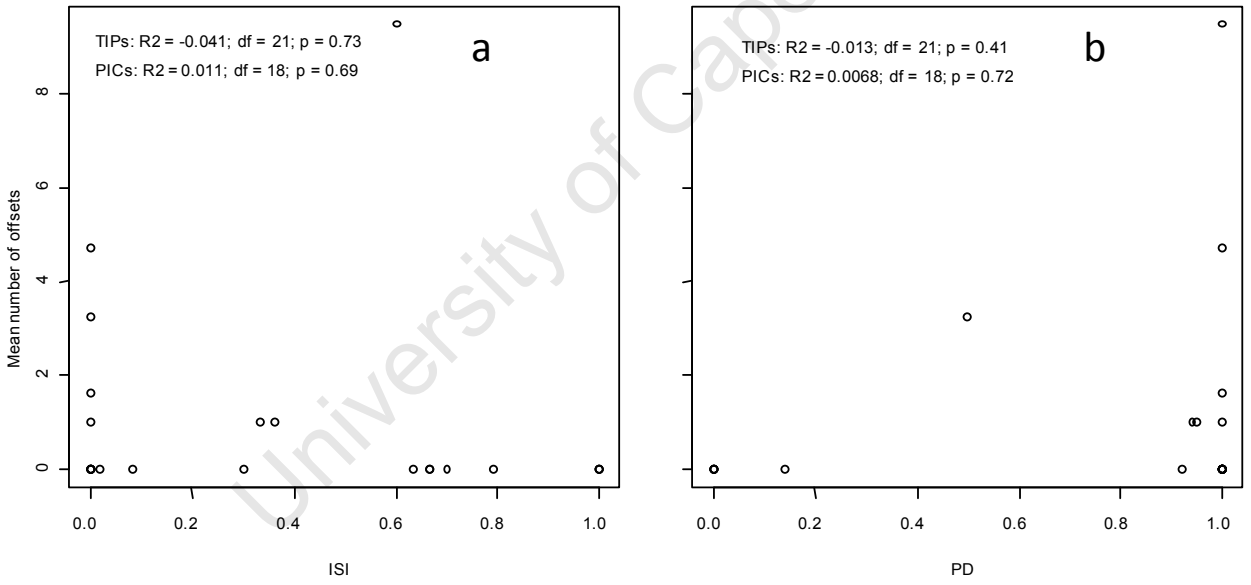

Figure 5. Associations between measures of self-compatibility and offset-production. ISI = index of self-incompatibility; PD = pollinator-dependence; mean offset number is calculated per offset-producing bulb.

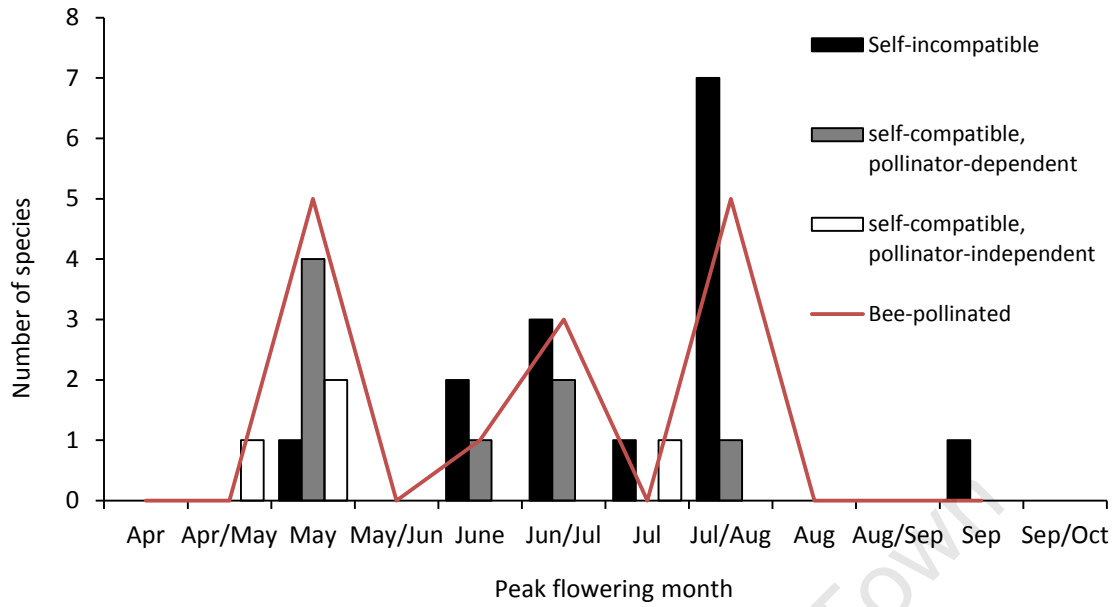

Figure 5. Distribution of peak flowering periods.

DISCUSSION

Opening paragraph

The results of this study show that sexual mechanisms of reproductive assurance are widespread in the *Lachenalia* species examined. Autonomous self-pollination was indeed found to be derived, only evolving in lineages which were already self-compatible. One species, *L. calcicola*, can be added to the three study species previously known to set seed in the absence of pollinators. Autonomous self-pollinators lack high spatial separation of male and female parts and produce less attractive floral displays than pollinator-dependent species. The role which offset-production plays in providing reproductive assurance is unclear, as this character state has been lost in self-incompatible and pollinator-independent lineages, but not in geitonogamous self-pollinators. Additionally, the hypothesized associations between autonomous self-pollination, flowering phenology and intraspecific polyploidy were not detected.

Sexual mechanisms of reproductive assurance

Ancestral character state reconstructions (Figure 1) indicate that self-compatibility is the ancestral character state amongst the species of *Lachenalia* studied, with state switches to self-incompatibility and even reversions amongst some of these self-incompatible species to self-compatibility. That species have been able to evolve self-incompatibility from an initial state of self-compatibility is surprising, given the complexity of the genetic controls of self-incompatibility and the rarity of their development in other cases. Given that transition to self-incompatibility is also hindered by the morphological and genetic changes accompanying self-compatibility, uncovering the nature of these mechanisms in *Lachenalia* would be of great interest.

Autonomous self-pollination

While autonomous self-pollination has not been conclusively shown to be associated with a reduction in the number of flowers per inflorescence, it does appear to be associated with shorter inflorescences and with low floral display, a metric which combines mean number of flowers per inflorescence and mean corolla diameter to produce a measure of attractiveness to pollinators (Goodwillie et al. 2010). As the metric only considers visual traits it may underestimate the attractiveness of visually underwhelming but strongly scented inflorescences; neither does it incorporate colour, the visible and ultraviolet spectra of which are known to play an important role in attracting pollinators

Pollinator limitation

Almost all species of *Lachenalia* occur in winter-rainfall areas; most of the species in this study found in the fynbos and Succulent Karoo biomes, and most for which pollinators have been identified are bee-pollinated. Kuhlmann (2009) found not only that the majority (46.3%) of South African bee species are endemic to winter rainfall regions, but that the fynbos and Succulent Karoo harbour the highest species diversity per unit area, up to 167 species per 2°×2° cell. Thus it seems that pollinator-limitation is unlikely to necessitate reproductive assurance; the alternative driver is mate-limitation.

Correlates of vegetative reproduction

Mate-limitation

Offset-production, rather than being less common in self-compatible lineages, was found to have been lost most often in self-incompatible species. If offset-production occurred only in self-compatible *Lachenalia* species, then it could be suggested that clonality causes mate-limitation by reducing the amount of outcross pollen received, thus making self-compatibility beneficial (Vallejo-Marin et al. 2010). Species that clone with offsets – which are not easily dispersed – should have ramets that are tightly packed, excluding other genets (Vallejo-Marin et al. 2010). If pollinators move randomly between individuals within such a population, then ramets in the interior of genets will receive more self-pollen (pollen that is genetically-identical to their own) than outcross-pollen (pollen produced by an individual of another genet). This theoretical association between clonal architecture and self-compatibility has, however, been little tested (Charpentier 2002; Vallejo-Marin et al. 2010).

All of the known intraspecific polyploid species included in the current study are self-compatible and offset-producing, but whether the association between polyploidy and self-compatibility is widespread amongst angiosperms is unknown, as few taxa have been studied (Mable 2004). Some authors have even suggested that inbreeding depression may be higher in tetraploids than in diploids, suggesting that selection for self-compatibility may be lower in the former than in the latter (Busbice and Wilsie 1966, Bennett 1976; both cited in Mable 2004).

Precipitation

Offset-production is also present, however, in some of the self-incompatible species. Selection for and against offset production may therefore be driven by needs other than reproductive assurance. Duncan (2005), working with a far more inclusive species set, noted that offset-producing species tend to occur in higher rainfall regions, whereas species with solitary bulbs are distributed almost entirely within more arid regions such as the Richtersveld, southern Namibia and Little Karoo. The almost complete lack of offset-production in water-limited regions suggests that the trait is selected against when water is scarce, whereas species in areas

of higher rainfall receive more water than they can store, and can thus afford to allocate some to offspring through the production of offsets. Seeds, being lighter, can be dispersed over larger distances, and, with less resource investment by the parent, can be produced in large numbers. But offsets are able to remain dormant through the dry summer, surviving on their nutrient and water stores, and begin growth at the onset of the winter rains. This may give them an advantage over seeds, which once germinated must have constant access to moisture. Rather than using all resources in exclusive seed-production or exclusive offset-production, doing both provides reproductive insurance should seed mortality be high.

Conclusion

Almost all the species studied exhibited some level of self-compatibility, vegetative reproduction, or both. Inferences about the relationships of these, and of the role of pollinator-limitation, are however constrained by sub-optimal support values for the phylogeny, the possible presence of paralogues, lack of knowledge of the pollinators of many species, and also of ploidy levels.

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