The evolution and persistence of polyploidy in *Oxalis obliquifolia* Steud. ex A.Rich. populations in Gauteng

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Declaration

I, *Damian Vaz de Sousa* declare that this thesis/dissertation, which I hereby submit for the degree *MSc in Plant Science* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Date: 17/11/2022

Summary

Background: Polyploidy is a major factor in the adaptation and speciation of many plant lineages. Many evolutionary factors may contribute to autopolyploid frequency within plant populations, including rates of new polyploid formation, the ability of new polyploids to establish successfully, long-term persistence of polyploids in the environment, and the ability of new polyploids to expand their range. Despite recent progress, there are still many questions regarding polyploid success, in spite of the challenges posed by minority cytotype exclusion, and relatively little is known about ploidy variation in the South African Flora.

Aims and objectives: *Oxalis obliquifolia* Steud. ex A.Rich. is notable for its large distribution range (from the Cape to Ethiopia) and high degree of morphological variability. The aims of this investigation were to document the occurrence of different cytotypes of *O. obliquifolia* across Gauteng Province, South Africa, and assess the impact of empirical data on theories that attempt to explain polyploid persistence in populations. The objectives of the study were: firstly, to sample individuals of *O. obliquifolia* across Gauteng and assess their cytotype using flow cytometry and chromosome squashes; secondly, to determine if there were differences in abiotic niches occupied by different cytotypes; thirdly, to determine the morphological and phenological traits associated with polyploidy; fourthly, to assess the degree of reproductive isolation between different cytotypes; and finally, to assess the degree of relatedness between individuals of different ploidy-levels across mixed-ploidy sites.

Methods: Over 320 samples from 25 sites were collected and cytotyped, using standard flow cytometric and ploidy confirmed using meiotic chromosome squashes. Individuals were mapped and abiotic variables assessed for correlations with cytotype distribution using GIS, climate data, field observations, soil data, and ordinations and PerMANOVAs. Different cytotypes (100 individuals, including diploids, tetraploids and hexaploids) were grown under identical conditions to assess the associations between polyploidy and morphology and phenology, and results were analysed using linear models and discriminant analyses. Reproductive isolation and frequency of polyploidisation were assessed using crossing experiments (1140 crosses, with different maternal cytotypes), as well as AMOVA analyses based on Internal Transcribed Spacer DNA sequences.

Results: Remarkably, six distinct cytotypes were identified, with over 50% of sites comprising multiple ploidies. Abiotic variables were not associated with cytotype distribution

possibly due to scale. The common garden experiment demonstrated a clear Gigas effect, which may confer a competitive advantage for polyploids over their smaller diploid progenitors. Larger flowers and differences in flowering phenology suggest pollinator interactions may play a role in enabling polyploid persistence. Crosses between cytotypes are possible under artificial settings, however DNA analysis suggests diploids and polyploids are reproductively isolated in the wild, and that polyploidisation is not a frequent enough event to explain the high levels of cytotype sympatry observed. Diploids and polyploids are behaving as separate species, despite high sympatry and non-zero potential inter-cytotype seed set. Tests on biotic interactions may provide insights into how polyploids have flourished in this system.

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GENERAL INTRODUCTION

i. Polyploidy, evolution and novelty

Mutation is the fundamental mechanism by which evolution occurs, and provides the most basic material on which natural selection can act (Dobzhansky, 1938; Carlin, 2011). There are three general categories of chromosome-level mutations, namely chromosome rearrangements (the alteration of the structure of chromosomes), aneuploidy and polyploidy (Pierce, 2017). Aneuploidy describes the condition whereby one or more chromosomes have been added or deleted, and such occurrences have been known to produce unstable, and often lethal results (Ehrendorfer, 1980; Guerra, 2008). In contrast, polyploidy, or Whole Genome Duplication (WGD), involves the addition of one or more sets of chromosomes, in other words a polyploidy is defined as the possession of more than two complete chromosome sets (Winge, 1917; Wendel, 2000; Tate *et al.*, 2005; Pierce, 2017).

Three primary types of polyploids are recognised, based on their origins (Stebbins, 1947): autopolyploids, allopolyploids and segmental allopolyploids. Autopolyploids are polyploid organisms "in which all of the chromosome sets are derived from the same species" (Stebbins, 1947; Lewis, 1980; McGrath and Lynch, 2012). Allopolyploids, on the other hand, are organisms that have multiple chromosome sets originating from different species (Stebbins, 1947; Grant, 1975; Kihara and Ono, 1926). And finally, segmental allopolyploids, which possess two or more copies of partially differentiated genomes (Stebbins, 1947; Levin, 2002). Other explanations for intraspecific variation in chromosome number include dysploidy and Beta-chromosomes. Dysploidy (sometimes also referred to as pseudoaneuploidy; Winterfeld *et al.*, 2018) refers to the alteration of the base chromosome number via chromosome rearrangements such as chromosome breakages (fissions) and chromosome fusions (Siljak-Yakovlev, 1996; Schubert and Lysak, 2011; Vallès *et al.*, 2011; 2012; De Storme and Mason, 2014).

Polyploidy has been widely acknowledged as an important factor in adaptation and speciation in many plant lineages (Blanc and Wolfe, 2004; Soltis and Soltis, 2009) and was first introduced as a mechanism of adaptation and speciation by Winkler (1916). Studies suggest that the majority of land plant genomes harbour some evidence of at least one polyploidisation event (Doyle *et al.*, 2008; McGrath and Lynch, 2012) in their evolutionary

histories. An early WGD event is known in the common ancestor of seed plants (Jiao *et al.*, 2011), as well as in the ancestral angiosperm (De Bodt *et al.*, 2005), which may be directly related to the rapid radiation of the angiosperms (De Bodt *et al.*, 2005). In addition to early polyploidisation events (such as in Vision *et al.*, 2000; Jaillon *et al.*, 2007; Barker *et al.*, 2008; Tang *et al.*, 2008; Tang *et al.*, 2009; Shi *et al.*, 2010; Chen *et al.*, 2022), polyploidy has been extensively recorded in many extant angiosperm species (Soltis and Soltis, 1993; Leitch and Bennett, 1997; Soltis, *et al.*, 2009; Wood *et al.*, 2009; Fawcett and Van der Peer, 2010; Wu *et al.*, 2020), including in speciose families such as the Asteraceae, where polyploidy occurs in all major clades, and which boasts an impressive number of ploidy levels, ranging from 2*n* to 48*n* (Semple and Watanabe, 2009). Furthermore, many angiosperm lineages with very small genomes, and/or few chromosomes, are derived from ancient polyploids, through a process of rediploidisation (Otto, 2007; MacKintosh and Ferrier, 2018), for example *Arabidopsis thaliana* (L.) Heynh., which is believed to be an example of a paleohexaploid (Blanc *et al.*, 2003).

Polyploids can arise by different mechanisms, such as somatic chromosome doubling (deWet, 1979; Ramsey and Schemske, 1998), but the general consensus is that unreduced gamete formation following meiosis is the most frequently occurring mechanism, which gives rise to the occurrence of higher ploidy-level cytotypes (Ahloowalia and Garber, 1961; Bretagnolle and Thompson, 1995; Suda and Herben, 2013). Unreduced gamete formation, as the model of the origin of polyploids was originally applied to autopolyploids, but is also a valid mechanism for the formation of allopolyploids (Suda and Herben, 2013).

It has been previously been suggested that allopolyploids are usually more stable than autopolyploids, in other words hybrid genotypes are more likely to become successfully established over the course of successive generations, due to the complete sets of parental chromosomes that enable appropriate pairing and segregation of chromosomes during meiosis (Ramsey and Schemske, 2002; McGrath and Lynch, 2012). However, later research has established that autopolyploids are more stable and persistent than previously thought and may in fact be capable of effective chromosome pairing, and thus capable of producing viable offspring (Soltis *et al.*, 2007; McGrath and Lynch, 2012). Recent data has also suggested that autopolyploids may play an important ecological and evolutionary role within natural populations of many species and that their frequency has previously been underestimated (Soltis *et al.*, 2007; Otto, 2007; Parisod *et al.*, 2010; Suda and Herben, 2013; Barker *et al.*, 2016).

There have been many views expressed on the potential evolutionary implications of polyploidy (Winge, 1917; Müntzing, 1936; Madlung, 2013). These range from theories that suggest polyploidy plays a significant role in the facilitation of rapid rates of evolution and diversification in many plant lineages (Ohno, 1970; Soltis and Soltis, 1993; 2000; Mayfield *et al.*, 2011), to the opposite view where polyploids are viewed as evolutionary dead ends, with little contribution to longterm diversification (Stebbins, 1950; Wagner, 1970; Levin, 1975; Mayrose *et al.*, 2011; Arrigo and Barker, 2012). Regarding the latter view, Wagner (1970) argued that polyploids are not important in plant lineage diversification or play a key role in plant evolution, since they are "blind alleys that go nowhere", and Stebbins (1971) described polyploidy as "a hindrance to the evolutionary success of higher plants". It was argued that while polyploids may be an important factor for plant diversity over shallow evolutionary timescales, they had little long-term evolutionary impact, since they were viewed as genetically depauperate (Stebbins, 1950; Wagner 1970; Soltis *et al.*, 2014*a*), and that the majority of evolutionary change was at the level of the diploid parents. Stebbins (1950) argued that the multiple genome copies resulted in masking both deleterious effects and beneficial mutations, and since polyploidy was often found to increase self-fertilisation, novel combinations of genes were rarely formed, thereby reducing the rate of adaptive evolution (Weiss-Schneeweiss *et al.*, 2013).

However, the former view, that polyploidy may constitute a more important factor in plant evolution has since gained broader acceptance (Soltis *et al.*, 2014*b*). This is due to advancements in molecular techniques that have revealed the occurrence of multiple polyploidisation events within many diverse plant lineages (Soltis and Soltis, 1993; 2000; Leitch and Bennett, 1997; Fawcett and Van der Peer, 2010). This lead to the realisation that recurrent polyploidisation potentially resulted in the possible maintenance and incorporation of higher levels of genetic variation, acquired from multiple diploid parent populations (for example in Soltis and Soltis, 2000; Tate *et al.*, 2005; Sampson and Byrne, 2011), which may offer certain advantages to polyploids.

Other authors have directly explored the advantages associated with being polyploid, many of which are derived from multiple gene copies (for example in Soltis and Soltis, 1993; Wendel, 2000; Gu *et al.*, 2003; Adams and Wendel, 2005; Lynch, 2007; Madlung, 2013). Most often, the ultimate fate of many gene copies is nonfunctionalisation (Lynch and Conery 2000; 2003) or silencing (Pikaard, 2001; Adams *et al.*, 2003; Wang *et al.*, 2004; Adams and Wendel, 2005). However, there are mechanisms by which duplicate genes may be preserved

(Hughes, 1994; Lynch, 2007; Innan and Kondrashov, 2010; Soltis and Soltis, 2012). One is neofunctionalisation, whereby genes diverge due to the acquisition of a novel function by the duplicate (Rastogi and Liberles, 2005; Conant and Wolfe, 2008; Futuyma and Kirkpatrick, 2017; Van Hieu, 2019). Subfunctionalisation occurs when duplicate genes diverge and each retains only a portion of the several functions of the original gene (Cusack and Wolfe, 2007; Futuyma and Kirkpatrick, 2017). Another mechanism involves the selection for increased gene product, due to changes resulting from dosage affects arise from duplicate genes (McGrath and Lynch, 2012). The overall preservation of these duplicate genes serves to increase the genetic repertoire among species' populations, which can have profound effects on the expression of plant morphology, physiology, ecology, and ultimately the ability of a newly formed polyploid individual to survive, and persist.

ii. Polyploid establishment and persistence

A newly formed polyploid individual, by necessity, must emerge into the context of an existing diploid parent population. This results in the immediate creation of a majority cytotype (the parent diploid) and a minority cytotype (the newly emergent polyploid). It is generally assumed that in such circumstances, the newly formed polyploid will become subject to the effects of Minority Cytotype Exclusion (Levin, 1975). The principle of Minority Cytotype Exclusion refers to the frequency-dependent process whereby the minority cytotype individual is constrained by a reproductive disadvantage, as a result of the compounded effects of high frequencies of between-cytotype crosses (since initially only the majority cytotype is available to breed with; Chrtek, *et al.*, 2017) and often substantial reproductive incompatibility between the majority and minority cytotypes (often manifesting as a triploid block; Bretagnolle and Thomson, 1995; Felber and Bever, 1997; Köhler, *et al.*, 2010). Therefore, it might be expected that Minority Cytotype Exclusion poses a major obstacle to polyploid evolution and long term persistence (Husband, 2000; Ramsey and Schemske, 2002; Otto, 2007; Fowler and Levin, 2016).

Polyploids are more likely to become established through the attenuation of the effects of Minority Cytotype Exclusion (Stebbins, 1950). There are four primary ways by which this may occur (Van Drunen and Friedman, 2022). Firstly, by way of a modification to the extent and potential for successful intracytotype and intercytotype reproduction. Secondly, the

challenge of limited available reproductive partners can be bypassed entirely by reducing dependence on sexual reproduction. The third way, is by the emergence of more polyploid individuals in the population, in other words, high rates of polyploidisation. And finally, by increasing the time that the newly formed polyploid is able to endure, and persist in the population.

Regarding the ability of newly formed polyploids to successfully reproduce, initially opportunities for reproduction are largely limited to crosses between the minority and majority cytotype. In the event that successful reproduction between cytotypes is possible, it may result in the production of higher-ploidy level cytotypes, through the creation of a triploid bridge (Burton and Husband, 2001; Yamauchi *et al.*, 2004; Peckert and Chrtek, 2006). The triploid bridge hypothesis views triploids as a key factor in the polyploidisation process, and it describes the process of tetraploid formation as a two step process, involving a triploid intermediary. The process is described as the initial fusion of reduced and unreduced gametes derived from diploid parents, resulting in a triploid offspring. This is followed by backcrosses between the triploid offspring and its diploid parents, or crosses among other triploids, resulting in the generation of tetraploids. Ultimately, this may allow for subsequent interploid crosses between tetraploids and triploids, thus increasing the prevalence of unreduced gametes in a population, thereby facilitating the increased likelihood of tetraploids becoming established, within existing diploid populations (Yahara, 1990; Ramsey and Schemske, 1998; Husband, 2004; Peckert and Chrtek, 2006), and potentially allowing for the generation of other higher-ploidy level cytotypes. However, in many cases polyploidisation events are frequently known to confer instant reproductive isolation between the diploid parents and the polyploid offspring (Thompson and Lumaret, 1992; Husband and Schemske, 2000; Husband and Sabara, 2004).

In the absence of the potential for successful intercytotype crosses, polyploid success would depend on the polyploid individual possessing a trait that either allows it to minimise competition with, or out-compete, the majority parent cytotype (Levin, 1975). In such circumstances, it has been determined that there are major roles for polyploids to achieve higher levels of fitness through increased potential for self-fertilization (Levin, 1975; Rodríguez, 1996; Mable, 2004; Rausch and Morgan, 2005), clonality and asexual/apomictic pathways of reproduction (Nakayama *et al.*, 2002; Yamauchi *et al.*, 2004; Hörandl and Hojsgaard, 2012; Hojsgaard *et al.*, 2014; Van Drunen and Husband, 2018; 2019; Hojsgaard and Hörandl, 2019; Spoelhof *et al.*, 2020), and potentially prolonged lifecycles/iteroparity

(Rodríguez, 1996) and perenniality (Gustafsson, 1948; Stebbins, 1950; Rodríguez, 1996; te Beest *et al.*, 2012; Chrtek *et al.*, 2017). Any of these different strategies would allow new polyploids to either pass on genetic material to subsequent generations, while avoiding the need for outcrossing sexual reproduction altogether, or to survive long enough until suitable homoploid mates arise.

The rate at which polyploidisation events occur in a lineage can have a major impact on the ability of polyploids to persist in populations. It has been argued that the rate of polyploidisation must necessarily exceed the rate of successful polyploid establishment in a population (Ramsey and Schemske, 2002). This suggests that polyploidisation events are potentially far more frequent than is apparent based on extant polyploids, particularly in those lineages known to already have multiple higher-ploidy level cytotypes. This may be due to the fact that once a polyploid has formed, it increases the amount of unreduced gametes in a population (Felber and Bever, 1997; Burton and Husband, 2001; Husband, 2004), thereby potentially facilitating the emergence of more polyploids. However, rates of polyploidisation have also been shown to be unevenly distributed, even in lineages where polyploidy is widespread. For example, Otto and Whitton (2000) observed that, within the angiosperms, polyploidy was more frequently occurring within dicots than within monocots.

In the case that polyploids are able to become established, the long-term persistence of the higher-ploidy level cytotype would depend on its ability to expand its distribution range. This would depend not only on the successful reproductive capacity of the higher-ploidy cytotype, as described above, but also on it's ability to successfully occupy a particular environmental or ecological niche, potentially different from that of its diploid parents. In those species that are self-incompatible, the conditions that would enable new polyploids to persist can be described by two distinct strategies (Levin, 1975; Fowler and Levin, 1984; Felber, 1991). The first strategy is that polyploid persistence could be achieved by avoiding direct competition with their diploid parents through niche separation/differentiation (Maceira *et al.*, 1993). Secondly, through the polyploid individual possessing a distinct competitive advantage over their diploid progenitors, which would in the long-term lead to single-ploidy (ie. not mixed) populations. (Maceira *et al.*, 1993).

Diploid parents and their polyploid offspring may possess fundamentally different requirements, both in terms of their physiology and biochemistry, thus resulting in them potentially occupying separate ecological niches (Lewis, 1980; Tal, 1980; Levin, 1983; Stebbins, 1985; Marchant *et al.*, 2016). This has been observed in many species for aspects of

both abiotic (for example, Borrill and Linder, 1971; Lumaret, 1985; Lumaret *et al.*, 1987) and biotic (for example, Lumaret, 1988; Lumaret and Barrientos, 1990) niche requirements. It has also been observed that polyploids may exhibit a higher degree of adaptability, than their diploid parents, which can sometimes manifest as an increased tolerance to abiotic stress factors (McIntyre, 2012; Allario *et al.*, 2013; Van de Peer *et al.*, 2021). Indeed, polyploidy has also been linked with invasiveness in some species, where it is largely seen as facilitating invasions of new habitats by the ability of polyploids to better adapt to, or tolerate, environmental pressures and stress (for example in Lafuma *et al.*, 2003; Mandák *et al.*, 2005; Treier *et al.*, 2009; te Beest *et al.*, 2012).

It has also been argued that a competitive advantage may be conferred on polyploids, due to direct changes in phenotype and/or morphology, associated with increased genome size. One direct effect of polyploidisation is that it results in an increase in cell size (Müntzing, 1936; Stebbins, 1971; Masterson, 1994). This has immediate consequences for the physiological traits of the plant that, as discussed above, may impact ecological niche requirements. The increased cell size is also often correlated with changes in morphology (te Beest *et al.*, 2012). In particular, polyploids are often observed to be larger and more vigorous, with larger floral structures and seeds (Garbutt and Bazzaz, 1983; Levin, 1983; Bretagnolle *et al.*, 1995; Segraves and Thompson, 1999). This increased size of the adult polyploid plant, and more vigorous seedlings, may facilitate enhanced competitiveness (Blossey and Nötzold, 1995; Jakobs *et al.*, 2004; te Beest *et al.*, 2012; Van de Peer, 2021), over its diploid parents (for example in *Dactylis glomerata* (Maceira *et al.*, 1993). It has been proposed that this enhanced competitiveness of polyploids is a crucial factor governing polyploid occurrence and patterns of distribution (Lumaret *et al.*, 1997).

Studies that assess changes in chromosome numbers and/or genome sizes, the mechanisms by which these emerge, their frequency of occurrence across lineages, and the relationships between different cytotypes and their morphological, ecological and reproductive traits, offer an effective approach to understanding the patterns of multiple cytotype occurrences and distributions among natural species populations.

iii. Polyploidy in sub-Saharan Africa

Global patterns of polyploid distribution suggest a latitudinal trend, with polyploid frequency increasing with higher latitudes. This pattern has been known for some time, particularly for the Northern Hemisphere (see Hagerup, 1931; Brochmann *et al.*, 2004; Martin and Husband, 2009). Recent studies at a global scale (Rice *et al.*, 2019) have robustly supported this pattern, and this suggests that climate, in particular temperature, may be one of the most influential factors influencing patterns of polyploid distribution. Tropical and subtropical regions have also been observed to be generally polyploid poor. It is notable from the information provided in Rice *et al.* (2019), that there is a general lack of available data on the occurrence and distribution of polyploids in sub-Saharan Africa, when compared with other regions around the world. One notable exception is the Greater Cape Floristic Region (GCFR) of South Africa. The GCFR, although highly species rich (Goldblatt, 1978; Linder, 2003; Manning and Goldblatt, 2012), has also been shown to have relatively low proportions of polyploidy (Oberlander *et al.*, 2016). It has previously been suggested that the relatively stable climate of the region may be the cause for general lack of polyploid plants (Dynesius and Jansson, 2000). However, evidence also suggests some lineages possess higher levels of polyploidy than others (for example see Goldblatt and Johnson, 1979; Krejčíková *et al.*, 2013*a*; 2013*b*; Rice *et al.*, 2014; Linder *et al.*, 2017).

Many *Oxalis* L. species found in the GCFR have been shown to include substantial ploidy variation across their distributions (see Heitz, 1927; Marks, 1956), with evidence suggesting a large number of different cytotypes have established and persist in existing populations. *Oxalis obtusa* Jacq., a widespread and highly variable species (Salter, 1944), was found to have seven distinct cytotypes (Krejčíková *et al.*, 2013*b*). Although little cytogeographic pattern was observed, there was some correlation between different cytotypes and environmental conditions, including vegetation type (where hexaploids were most common in the Fynbos biome, while tetraploids were most common in the Succulent Karoo biome) and precipitation (Krejčíková *et al.*, 2013*b*). Similar cytotype distribution patterns have also been observed in *Oxalis purpurea* L., with at least five cytotypes identified (Becker *et al.*, 2022). Polyploidy has also been linked to invasiveness in *Oxalis pes-caprae* L., which is native to the GCFR, but has also become a problematic invasive species in many other parts of the world (Randall, 2012; Sanz Elorza *et al.*, 2004). Evidence suggests that only diploid, triploid and tetraploid cytotypes are found across indigenous populations, whereas only tetraploid and pentaploid cytotypes have

been recorded across their the invaded range (Krejčíková *et al.*, 2012). Unpublished data suggests that this pattern of remarkable ploidy diversity is common throughout GCFR *Oxalis* (R. Schmickl and K. Oberlander, pers.comm.).

However, despite recent progress, there is a notable paucity of information and data regarding ploidy variation in the flora of subtropical southern Africa (Rice *et al.*, 2019) outside the GCFR in general and non-GCFR African *Oxalis* in particular. To date there has only been a limited number of investigations into the occurrence of polyploidy in non-GCFR African *Oxalis*, and there is much that is still unclear regarding the frequency and distribution of whole genome duplication events within these species populations.

iv. Study species

Oxalis obliquifolia Steud. ex A. Rich., which belongs to a predominantly GCFR clade (Oberlander, 2011) and is a close relative of *O. obtusa*, has the largest distribution range of all African *Oxalis*, extending throughout the eastern, summer-rainfall regions of South Africa (Exell *et al.*, 1963), all the way northward, through eastern Africa to Ethiopia (Raimondo *et al.*, 2009), Eritrea (Edwards *et al.*, 2000) and Sudan (Darbyshire *et al.*, 2015). This is unique among southern African *Oxalis* and makes this species a particularly promising candidate for

Figure I: Selected images of *Oxalis obliquifolia* found across different sites (**A-** Prime View, Olifantsfontein; **B-** Legends Mountain Bike Trails, Pretoria East; **C-** Hennops Hiking Trails, Magaliesburg) in Gauteng.

studying the occurrence of polyploidy and geographic patterns of cytotype distribution. Furthermore, there is evidence to suggest that it also harbours substantial ploidy variability (J. Suda, unpublished data), and the lack of close relatives of *O. obliquifolia* over the vast majority of its range implies that polyploids in this species are most likely autopolyploids.

This species occurs abundantly in grasslands and wooded grasslands (Exell *et al.*, 1963), with the most recent assessment identifying its population risk-level as "Stable" and of "Least Concern" (Raimondo *et al.*, 2009). From an ethnobotanical perspective, it has been reported that the leaves are known to be harvested and eaten (both cooked and fresh) in northwestern Ethiopia (Abera, 2022).

Like all other members of the southern-African *Oxalis* lineage (Gebregziabher, 2004; Oberlander *et al.*, 2011), *Oxalis obliquifolia* (Figure I) is a bulbous perennial with a vertically growing, subterranean rhizome, which grows from an ovoid shaped bulb, covered with a dark brown tunic (Salter, 1944). The leaves are borne on the emergent rhizome, in a rosette arrangement at the soil surface. The leaves are trifoliolate, with the petiole wider at the base than at the apex, and with trichomes occurring along its entire length (Salter, 1944). Vegetative propagation occurs via bulbils that grow from the underground rhizome, which grow to establish clusters of clonal adult plants.

It flowers during the rainy months of the austral summer, and enters a state of dormancy by dying back to the subterranean bulb during the colder, dry winter periods. Flowers are solitary on erect peduncles (technically a reduced, single-flowered inflorescence), that are often longer than the surrounding petioles. Petals range from white to bright-pink, with a yellow corolla tube, and often bear distinct markings that may be nectar guides (UV reflectance has not been assessed for this species). The fruit is a globose, loculicidal, five-parted capsule terminated by the persistent styles. Sexual reproduction in *O. obliquifolia* is influenced by heterostyly (tristylous flower morphs, where legitimate crosses between plants requires pollen transfer from flowers with long-, mid-, or short-level anthers to flowers on the corresponding long-, mid-, or short styles, respectively; Barrett, 1990; Krug *et al.*, 2012), as well as generalist insect pollination (specific pollinators for this species and close relatives are unknown). Short-distance ballistic seed dispersal is characteristic of this species, on the scale of several meters. Seeds are small with faint markings/ribbing and with a waxy cuticle over the seed coat.

v. Scope of investigation, aims and objectives

Research Question:

How do polyploids persist within local populations of *Oxalis obliquifolia* in Gauteng?

The aims of this investigation were to document the occurrence of different cytotypes of *Oxalis obliquifolia* across Gauteng province, South Africa, and to assess the assess the impact of empirical data on proposed theories that may explain the persistence of polyploids in species populations.

In order to achieve these aims, four key questions were investigated:

1. Do different cytotypes co-occur, and do they occupy different abiotic niches?
2. Is polyploidy associated with changes in plant morphology and/or phenology, in this system?
3. Is there evidence of higher or lower reproductive isolation between different cytotypes?
4. How frequently do polyploidisation events occur in this system?

Each of these questions is addressed as part of the different chapters in the following document, each with its own experimental procedure and statistical analyses, and discussion. The first chapter identifies and maps cytotypes across the study area, and includes assessing whether, or not, there are any differences in abiotic variables associated with the distribution of different cytotypes. The second chapter focuses on possible character differences, both morphological and phenological, between cytotypes. Finally, the third chapter focuses on the degree of reproductive isolation between cytotypes, and frequency of polyploidisation. Due to the structure of this thesis there is a degree of repetition in terms of references and discussion points throughout the different chapters, as each chapter provides a general context for the specific aspects addressed under each section, which pertains to each particular direction of enquiry. It is intended that individual chapters provide enough information to stand-alone and potentially be published individually, while still forming part of one larger comprehensive investigation into the main research question.

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CHAPTER 1: Cytotype identification and cytogeography of *Oxalis obliquifolia* in Gauteng

1.1. Introduction

Polyploidisation events can have profound effects on plant physiology, and thus plant ecology (Levin, 2002; Ramsey and Ramsey, 2014; Duchoslav *et al.*, 2020). The generation of many duplicate gene copies (genetic redundancy), offers the potential to evolve novel or slightly varied functions that can facilitate changes in the expression of genes in higher-ploidy level cytotypes (Adams, 2007; Yoo *et al.*, 2014; Jiao and Patterson, 2014; Saminathan *et al.*, 2015; Coate *et al.*, 2016; Gallagher *et al.*, 2016). This can result in marked changes to phenotype (see Chapter 2; Garbutt and Bazzaz, 1983; Levin, 1983; Lumaret, 1988; Bretagnolle *et al.*, 1995; Balao *et al.*, 2011; te Beest *et al.*, 2012), subsequently having instantaneous consequences for polyploid ecology (Ramsey, 2011; Hahn *et al.*, 2012; Ramsey and Ramsey, 2014; Gallagher *et al.*, 2016; Van de Peer, 2021), selection (Bretagnole and Thompson, 1996; Jiang *et al.*, 1998; Otto and Whitton, 2000; Otto, 2007; Balao *et al.*, 2011), their response to environmental conditions (Adams and Wendel, 2005; Lynch, 2007; te Beest *et al.*, 2012; Duchoslav *et al.*, 2020) and ultimately their patterns of cytotype distribution. Studies that describe the structure of cytotype diversity and distribution within species populations are critical for advancing our understanding of the evolutionary factors that impact and determine the successful establishment of cytotypes.

Competition between individuals is a prominent factor in the determination of ecological niches of species (Berendse, 1983; Schwinning and Kelly, 2013), and can likewise be applied to cytotypes (as they can be considered as distinct taxa; Suda *et al.*, 2007*a*). Since polyploids, by necessity, ultimately emerge from within existing diploid-parent populations, they must overcome the challenge posed by Minority Cytotype Exclusion (see Introduction Chapter; Levin, 1975). Once established, neopolyploids are then further confronted by the prospect of competition with their diploid parents (Karunarathne *et al.,* 2018), which are already present in larger numbers (Baack, 2005) and already occupying available niche space. Neopolyploids can either compete directly with, and attempt to out-compete, their diploid parents to survive, or they must avoid direct competition by ecological and niche differentiation (Hegarty and Hiscock, 2008; Raabová *et al.*, 2008; Treier *et al.*, 2009; Parisod *et al.*, 2010; Zozomová-Lihová

et al., 2015), which in some instances can be achieved through spatial/habitat segregation (Levin, 2003; Duchoslav *et al.*, 2020).

Competition avoidance may be accomplished by ecological displacement (the divergent evolution of ecological traits, as a result of selection, to avoid competition by acting on traits associated with the use of particular resources; adapted from Pfennig and Pfennig, 2009), and/or spatial segregation (Fowler and Levin, 1984; 2016; Van Dijk and Bijlsma, 1994; Ramsey and Schemske, 1998; Levin, 2003; Baack, 2004; Schönswetter *et al.*, 2007; Rieseberg and Willis, 2007; Sonnleitner *et al.*, 2010; Husband *et al.*, 2013; Karunarathne *et al.*, 2018). Niche differentiation and spatial segregation, are major factors that enable neopolyploids to expand their distributions (Fowler and Levin, 2016) and can often be viewed as a consequence of ecological differentiation along abiotic and/or biotic (see Chapter 2) environmental gradients (Endler, 1977; Johnson *et al.*, 2003; Brito *et al.*, 2016).

Changes to niche occupancy and requirements are well documented in polyploid plants, with habitat segregation being common in many polyploid complexes (for example in Lumaret *et al.*, 1987; Johnson *et al.*, 2003; Stuessy *et al.*, 2004; Hülber *et al.*, 2009). Polyploids can inhabit conditions at the same, or even beyond, the environmental tolerance of their diploid progenitors (see Hagerup, 1932; Soltis and Soltis, 1995; 2000; Soltis *et al.*, 2007; Kearney, 2005; Parisod *et al.*, 2010; Weiss-Schneeweiss *et al.*, 2013; Diallo, *et al.*, 2016; Fox *et al.*, 2020; Baniaga *et al.*, 2020), thus resulting in polyploids often possessing broader environmental tolerance, and facilitating ecological flexibility (Adams and Wendel, 2005; Dubcovsky and Dvorak, 2007; Lynch, 2007; Fawcett *et al.*, 2009; McIntyre, 2012; Madlung, 2013; Diallo, *et al.*, 2016; López-Jurado *et al.*, 2019). In other words, many polyploids not only inhabit harsher environments, but are also often better adapted to respond to abiotic environmental fluctuations.

Polyploid establishment and its association with increased environmental stochasticity (Leitch and Leitch, 2008; Oswald and Nuismer, 2011; Duchoslav *et al.*, 2020) suggests that polyploids may possess the ability to better colonise new environments (Baack, 2005; Treier *et al.*, 2009), and an increase in invasion potential (Pandit *et al.*, 2006; 2011; te Beest *et al.*, 2012; Rosche *et al.*, 2016). Additionally polyploids have been observed to be better equipped to endure extremes in abiotic factors, particularly in the context of extremes in temperature and rainfall conditions, often described in relation to latitude (Stebbins, 1984; Brochmann *et al.*, 2004; Burnier *et al.*, 2009; Rice *et al.*, 2019), elevation (Schinkle *et al.*, 2016; Dai *et al.*, 2020), and

environmental aridity (Ramsey, 2011; Liu *et al.*, 2011; Deng *et al.*, 2012; Manzaneda *et al.*, 2012; Duchoslav *et al.*, 2020).

The interactions between different cytotypes, and the results of direct competition and competition avoidance interactions between diploids and polyploids, can result in complex large- and small-scale distribution patterns (Stebbins, 1985; Thompson and Lumaret, 1992; Petit *et al.*, 1999; Buggs and Pannell, 2007; Kolář *et al.*, 2009; Martin and Husband, 2009; Trávníček *et al.*, 2011; Husband *et al.*, 2013; Kolář *et al.*, 2017). This suggests that patterns of cytotype distribution are a consequence of complex ecological processes and interactions. When polyploids initially arise, by necessity they occur in sympatry with existing diploid populations. While this pattern may change over time, there are instances where sympatric cytotype occurrence has endured and continues to persist (for examples see Husband and Schemske, 1998; Suda *et al.*, 2007*b*; Trávníček *et al.*, 2011). However, it is often the case that following the emergence of neopolyploids, cytotype distribution expansion or shrinkage can result in parapatric (cytotypes have distinct distributions that abut one another, with sometimes some small overlap) or allopatric (completely mutually exclusive and disjunct) distribution patterns (Krejčíková *et al.*, 2013*a*). It is also often the case that where cytotypes are observed to co-occur, they are in fact part of contact zones between larger parapatric cytotype distributions (Lexer and van Loo, 2006; Duchoslav *et al.*, 2010; Šafářová *et al.*, 2011; Castro *et al.*, 2012; Duchoslav *et al.*, 2020).

The study of geographic distributions of polyploids, in comparison to their diploid parents, can potentially provide valuable insights into the dynamics of polyploid population biology. This includes insights into factors that influence the patterns of cytotype distribution, such as environmental factors and habitat separation, and the development of larger polyploid complexes (Lo *et al.*, 2009). In particular, regions of cytotype co-occurrence at the local scale can offer unique opportunities to investigate intercytotype interactions, and assess the evolutionary forces that influence polyploid persistence in natural populations (Lewis and Suda, 1976; Burton and Husband, 1999; Krejčíková *et al.*, 2013*a*).

In this study, standard flow cytometric techniques, in addition to meiotic chromosome squashes, were used to assess cytotype variation among populations of *Oxalis obliquifolia*. In particular, the following questions were investigated: (1) What is the degree of cytotype diversity of *O. obliquifolia* across Gauteng Province? (2) What is the extent to which cytotype co-occur, or are they spatially segregated, in Gauteng? (3) Are abiotic variables correlated with cytotype distribution/occurrence?

1.2. Materials and Methods

Field sampling

A total of 28 sites across Gauteng Province were selected for investigation, which were identified based on documented occurrence data available on the Global Biodiversity Information Facility (GBIF, 2021: https://www.gbif.org/species/3627864) and iNaturalist (2021: https://www.inaturalist.org/taxa/591238-Oxalis-obliquifolia), and covering an area of approximately 9 500 km^2. Fresh leaf material from 10 to 15 individuals per site was harvested, during the summer growing season, for analysis and cytotype identification. In order to avoid sampling individuals of the same genet (clonal individuals arising asexually from bulbils) and to collect individuals across a larger area for each site, individuals were sampled a minimum of 35 meters apart from one another, and different flower morphs (long-, middle- and short-styled) were identified and included whenever possible. All occurrences and sample materials were recorded with coordinate data (with an accuracy of between 8m and 12m) for later mapping and analysis. Voucher specimens for each site were collected and deposited in the H.G.W.J. Schweickerdt Herbarium (University of Pretoria, PRU; Appendix 1A and 1B).

Flow cytometry

DNA ploidy levels were identified using relative fluorescence intensities of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei using standard flow cytometric techniques (adapted from the procedure described by Krejčíková *et al.*, 2013*b*). Fresh leaf material from each individual was analysed within 3 days of collection, and stored under refrigerated conditions (4 degrees Celsius). A two-step procedure using buffers Otto I (0.1M citric acid, 0.5% Tween 20; Otto, 1990) and Otto II (0.4M $Na_2HPO_4.12H_2O$) was used, where tissues were co-chopped together with an equal amount of an internal reference standard. This was done using a sharp razor blade in a Petri dish, which contained 1 mL of the Otto I buffer. In this case, *Oxalis articulata* was selected as the internal standard based on the availability of approximate genome size information from Vaio *et al.* (2016), and it was obtained from the Manie van der Schijff Botanical Gardens at the University of Pretoria. The suspension with co-chopped sample and internal standard was then filtered through a 30 µm mesh into a sample tube, and allowed to incubate at room temperature for at least 20 minutes. Subsequently, 1 mL of Otto

II buffer along with 10 µL DAPI per mL and 2 µL β-mercaptoethanol per mL was added to the solution, and allowed to incubate for 10 minutes to facilitate nuclei staining. Relative fluorescence of at least 5000 particles were captured using a CyFlow Space cytometer (Sysmex Europe GmbH), housed in PRU, equipped with a UV laser (wavelength set at 352nm; Doležel *et al.*, 2007) as the light excitation source. The resulting fluorescence histograms were analysed using 'FloMax®' software (version 2.4, Sysmex Partec GmbH). Samples were re-analysed in instances where a coefficient of variation (CV) value for any peak was above 5%. In some cases CV's of < 5% could not be achieved; in these cases, if after three separate runs the resulting peaks were found to consistently lie within a range of values associated with a particular genome size, then that individual was assigned to that cytotype.

Chromosome counts

The chromosome numbers of diploid and tetraploid individuals were confirmed utilising meiotic chromosome squash techniques, based on the approach described by Windham *et al.* (2020). Sample material was collected from multiple diploid and tetraploid individuals, as identified using flow cytometry. In order to ensure the presence of anthers at the required stage of meiosis a variety of flowers at different stages of development, erring towards the younger material, were sampled. Samples were fixed using a freshly prepared 3:1 95% ethanol : glacial acetic acid solution ("Farmer's fixative", stored on ice before and after use). After 24 hours the fixative was decanted and replaced with 70% ethanol, and stored in a −20°C freezer until further use. Samples were then placed onto a clean glass Petri dish and submerged in 70% ethanol to prevent drying out (adding more during the process as needed). Using a dissecting microscope, anthers were removed from the immature buds and broken open and isolated using a dissecting needle tip. A clean microscope slide, with a droplet of dilute acetocarmine stain, was then placed under a dissecting microscope. The isolated anther material was transferred into the droplet and further isolated without being allowed to dry out. A small droplet of full-strength acetocarmine was then added and the dissected anthers mixed into the stain. The anthers were crushed/mashed using the dissection needle positioned almost horizontally, until the majority of the sample was homogenised and individual cells were dispersed throughout the stain droplet. Excess tissue material was then removed, with the final droplet size, containing the individual cells and anther material, no more than 1 cm in diameter. A single droplet of Hoyer's solution (Anderson, 1954),

approximately equal in volume as the acetocarmine droplet, was added and mixed thoroughly. Under a dissecting microscope, the cleaned cover slip was lowered into position and gently tapped with the dissecting needle to removed the bubbles, and excess liquid. The sample was subsequently squashed vertically for about 15 seconds, gently released, then squashed for another 15 seconds on alternate corners, and in the centre of the cover slip. Excess Hoyer's solution was carefully removed using a wipe with 70% ethanol and cleaned. Countable chromosomes were then identified using a Nikon Eclipse E200 light microscope equipped with a mounted Nikon E950 digital camera, manufactured by Tochigi (Nikon Corporation, Japan).

Mapping and data collection

A total of 320 individuals with known cytotypes were mapped using ArcGIS Pro (GIS software; Version 10.0: Environmental Systems Research Institute, Inc., 2010) across 25 sites. GIS layers were then used to extract values to each coordinate point for specific abiotic variables relating to: climate, topography, and underlying geology. Climatic data (mean annual precipitation, minimum temperature during mid-winter (July), and maximum temperature during mid-summer (January)) were obtained from the WorldClim 2 data set (1970–2000; version 2.1) at 30 arc-s resolution (approximately 1 km^2) (Fick and Hijmans, 2017). Topographical variables were obtained by retrieving elevation data from the Shuttle Radar Topography Mission (SRTM; Jarvis *et al.*, 2008), at 30 arc-s (approximately 1 km^2) resolution, and subsequently this data was used to calculate slope and northness utilising the Slope and Aspect tools, respectively, in ArcGIS Pro. Geological data was obtained using the Chronostratigraphic map (created by the Council for Geoscience of South Africa) shapefile available for download through the Esri online portal (https://www.arcgis.com/home/item.html?id=739c8b22b99b47bb81c2bed660d6c5de). Additionally, microclimate variables relating to exposure (sun or shade conditions) and soil were also obtained for each individual. Soil samples were collected (about 50ml by volume) and thoroughly air dried before being analysed. During the period between soil sample collection and analysis, samples were stored in air tight containers (sealed immediately after air drying) and kept below -20°C, in line with standard practice (International Organization for Standardization; ISO 18512, 2007). Due to cost constraints detailed soil features such as pH, Nitrogen content, and Phosphorus content, could not be included in the study. However, to include at least some soil variables, simple

assessments of soil texture were included. Soil characterisation was done by separating the coarse fraction from the soil fraction (by using a 2mm sieve) and calculating the percent of coarse material by total dry mass. The soil fraction was then further characterised by texture following the ribbon method, as described by Natural Resources Conservation Service, United States Department of Agriculture (https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/edu/?cid=nrcs142p2_054311), which is a modified approach based on the methods described by Thien (1979) for soil texture classification (see Appendix 1C).

Statistical analysis

A Multiple Factor Analysis (MFA, Appendix 1D) using the FactoMineR package (Lê *et al.*, 2008), which combined a Principal Component Analysis (PCA) with a Multiple Component Analysis (MCA), was conducted in R version 4.2.0 (R Core Team, 2022). This allowed both continuous and categorical variables to be assessed for explanatory power in identifying the specific cytotype groups using abiotic conditions as predictor variables (10 abiotic variables in total, Appendix 1E). Statistical support for abiotic variable associations between cytotypes and sites were determined using Gower's distance (Gower, 1971) with the daisy() function in the cluster package (), and by using a PerMANOVA analysis (Appendix 1D) with the adonis() function in the vegan package (Oksanen *et al.*, 2022). Prior to the PerMANOVA analysis, traits were assessed for autocorrelation (Appendix 1D) using Pearson's correlation coefficient and the cor() function (R Core Team, 2022), and correlated traits were excluded, using a degree of correlation number of |0.7| as a cut-off for identifying strong correlations. Elevation was found to be highly correlated with both maximum temperature (|r| = 0.94) and minimum temperature (|r| = 0.72) were found to be highly correlated with one another, and so only elevation was retained for analysis.

1.3. Results

Cytotype identification

A total of 320 individual specimens of *Oxalis obliquifolia* from across Gauteng Province were assessed using standard flow cytometry techniques, and classified according to their relative genome size (measured against the internal standard, *O. articulata* (Table 1.1; Figure 1.1A-D), with an approximate genome size of 2C-x = 0.91 pg (based on Vaio *et al.*, 2016). Of those,

Table 1.1: Results of flow cytometric analysis of *Oxalis obliquifolia* samples

Ploidy level	Relative genome size (mean ± s.d.)*	n	Number of sites encountered
2x	0.849 ± 0.039	53	10
4x	1.621 ± 0.048	137	21
5x	1.944 ± 0.043	4	3
5x+	2.197 ± 0.037	2	1
6x	2.514 ± 0.068	55	9
8x	3.266	1	1

* Calculated as a ratio of sample to internal standard (sample/standard); Internal standard = *Oxalis articulata* (2C-x = 0.91 pg)

255 individuals were deemed to have good (below 5%) CVs, and were used for the construction and identification of cytotype categories/associated values. The mean CVs for the sample (G1) fluorescence peaks was 4.19 % (range 2.38 – 4.99 %). Six distinct cytotypes were identified (Figure 1.1E; Table 1.1), including diploids (2x; relative genome size = 0.849 ± 0.039), tetraploids (4x; relative genome size = 1.621 ± 0.048), pentaploids (5x; relative genome size = 1.944 ± 0.043), hexaploids (6x; relative genome size = 2.514 ± 0.068) and octoploids (8x; relative genome size = 3.266), and possibly an instance of aneuploidy (5x+; relative genome size = 2.197 ± 0.037). The mean relative monoploid genome size (1Cx-value; mean ± s.d.), for the three majority cytotypes, was found to be approximately 0.38 ± 0.009 pg.

In order to verify the flow cytometry results, meiotic chromosome squashes were performed using sample material harvested from individuals assumed to be diploids (20 individuals) and tetraploids (25 individuals). Chromosome counts for both diploids (Figure 1.2A) and tetraploids (Figure 1.2B) were determined to be 2n = 14 and 4n = 28 respectively, yielding a base chromosome number of n = 7, for the species *Oxalis obliquifolia*, while also confirming the accuracy of the relative genome sizes determined using flow cytometry. Chromosomes were mostly metacentric to submetacentric in structure (Appendix 1F).

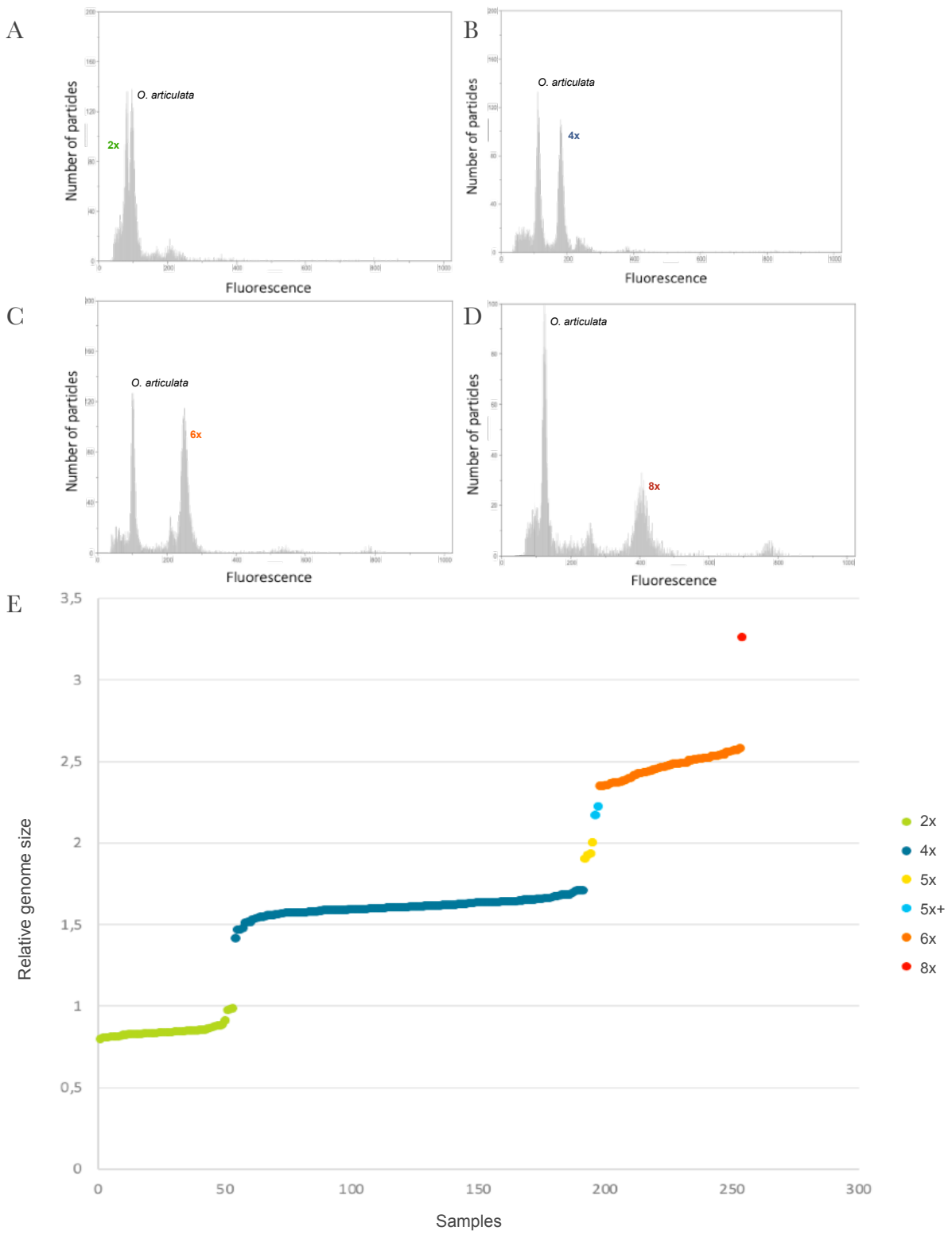

Figure 1.1: Selected fluorescence histograms showing different relative genome sizes (**A-** diploid, 2n; **B-** tetraploid, 4n; **C-** hexaploid, 6n; and **D-** octoploid, 8n) of *Oxalis obliquifolia* individuals, compared to the internal standard *O. articulata* (2C-x = 0.91 pg). **E-** Relative genome sizes of 255 individual *Oxalis obliquifolia* plants, with good CV values (below 5%), collected from across Gauteng province, South Africa, with 6 distinct cytotypes identified.

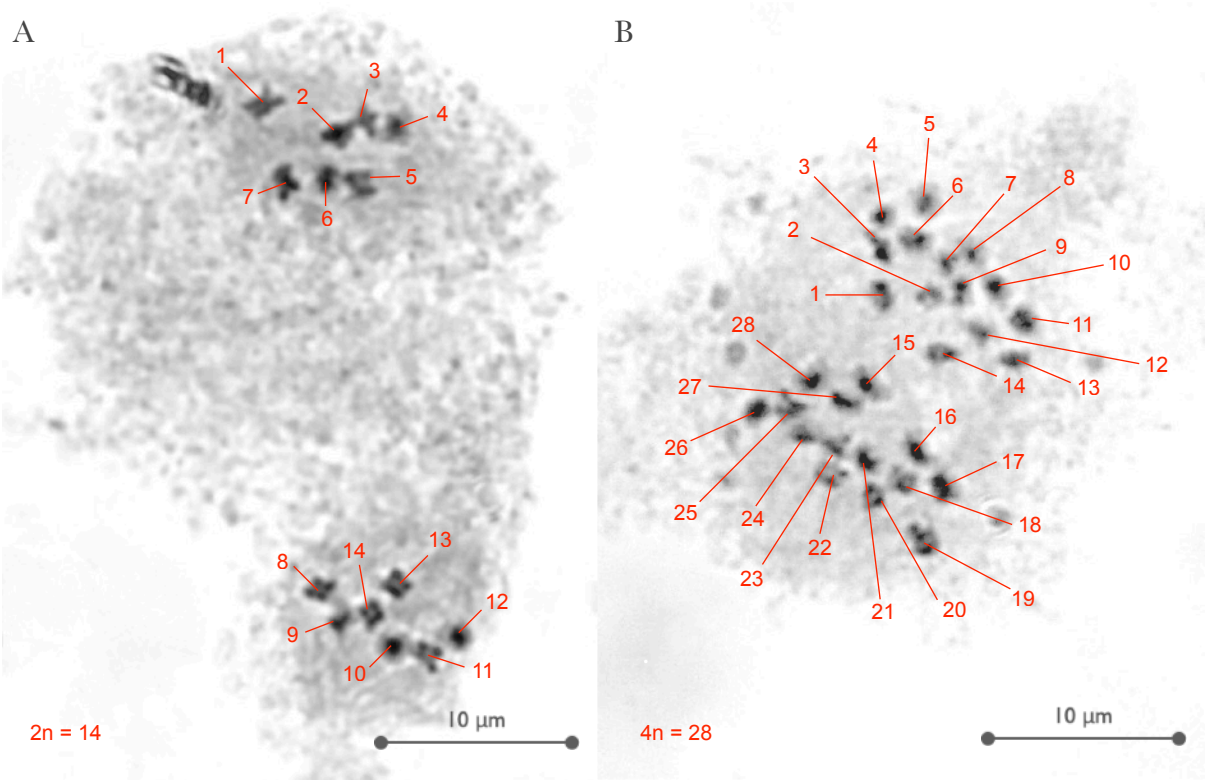

Figure 1.2: Meiotic chromosome squashes and chromosome counts in pollen mother cells of two of two *Oxalis obliquifolia* individuals (**A-** a diploid individual; **B-** a tetraploid individual), and chromosomes stained with acetocarmine solution and viewed under a light microscope.

Cytotype mapping

In total, 28 sites were investigated for the occurrence of *O. obliquifolia,*, covering an area of approximately 9500 km^2. In total 320 individuals were mapped across Gauteng (Figure 1.3A) and out of the 28 sites investigated, three had no *O. obliquifolia* individuals. Remarkably, over half (fifteen localities) of the remaining sites (Figure 1.3B) were found to have mixed-ploidy populations, with the remaining sites being either uniformly diploid (two sites), tetraploid (seven sites) or hexaploid (one site, Suikerbosrand Nature Reserve). These three cytotypes made up the largest portion of individuals encountered (Figure 1.3C), with the remaining individuals comprising minority cytotypes, including pentaploids and octoploids. Tetraploids were found to be the most commonly encountered cytotype, making up over half of all the individuals assessed.

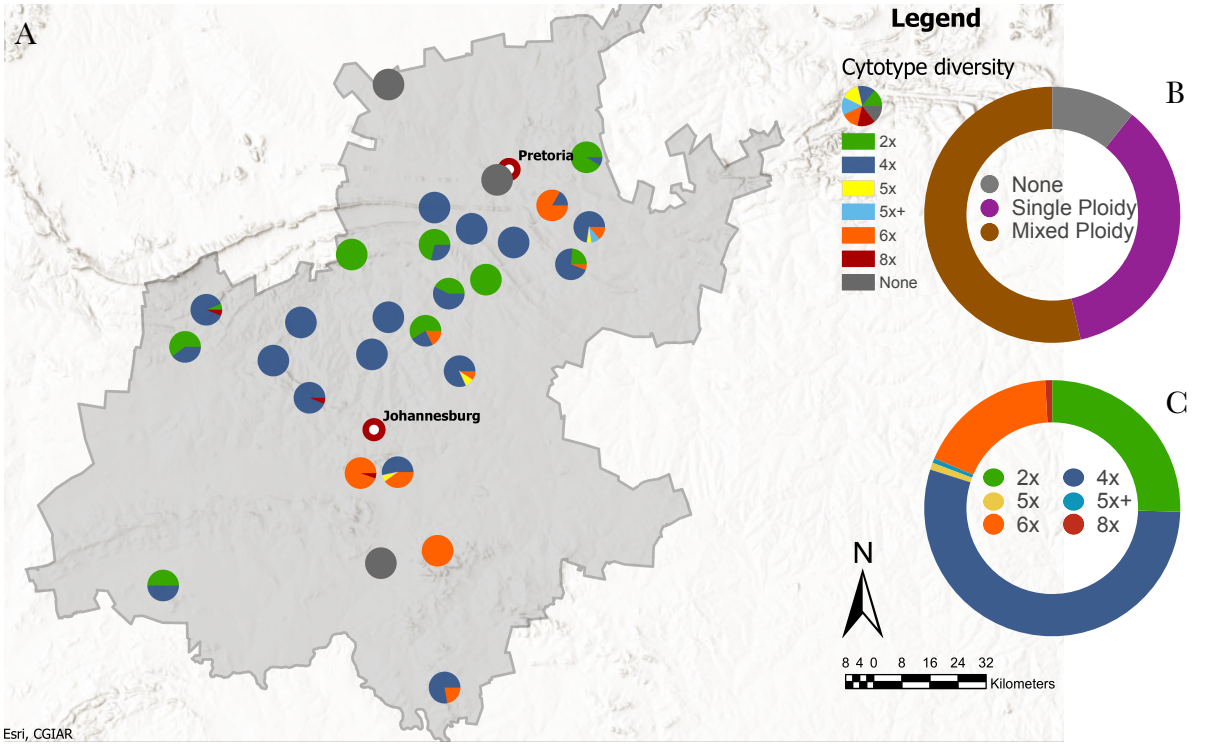

Figure 1.3: A- The cytogeography of *Oxalis obliquifolia* across Gauteng Province (indicated in grey), with the proportion of different cytotypes identified at each locality. **B-** Proportion of sites with no *O. obliquifolia* (grey), only one cytotype (purple) or mixed cytotype (brown) individuals. **C-** Total proportion of cytotypes encountered across Gauteng Province.

Environmental niche differentiation

The MFA showed a substantial degree of overlap between cytotypes (Figure 1.4). Based on the ten sampled abiotic variables, clusters based on cytotypes are not identifiable, with tetraploids overlapping with both diploids and hexaploids (Figure 1.4A). Total explanatory power of the MFA for the first two axes was very low, accounting for only 19.98% of the variation. It was further found that only after reaching dimension 8 (out of a total of 34 dimensions), did the cumulative percentage of variation reach 50%. This strongly suggests that abiotic variables do not significantly contribute to determining the cytotype distribution patterns observed in this system, and at the scale of this investigation. Characters with the largest contribution to the construction of dimension 1 on the x-axis of the MFA were minimum temperature and maximum temperature (Figure 1.4 D; Appendix 1G). These variables are strongly correlated with latitude suggesting site, and not cytotype, is better described by these data. It is worth noting that the supplementary variable "Site", and not "Cytotype" was most strongly associated with the variation accounted for by dimensions 1 and 2 (Figure 1.4C), which implies very little association between the abiotic variables

included in this study and the distribution of cytotypes encountered in the field. Indeed, individual clusters suggest local conditions are more informative than broad-scale variables., as shown in Figure 1.4B. This observation was supported by the statistical analysis results

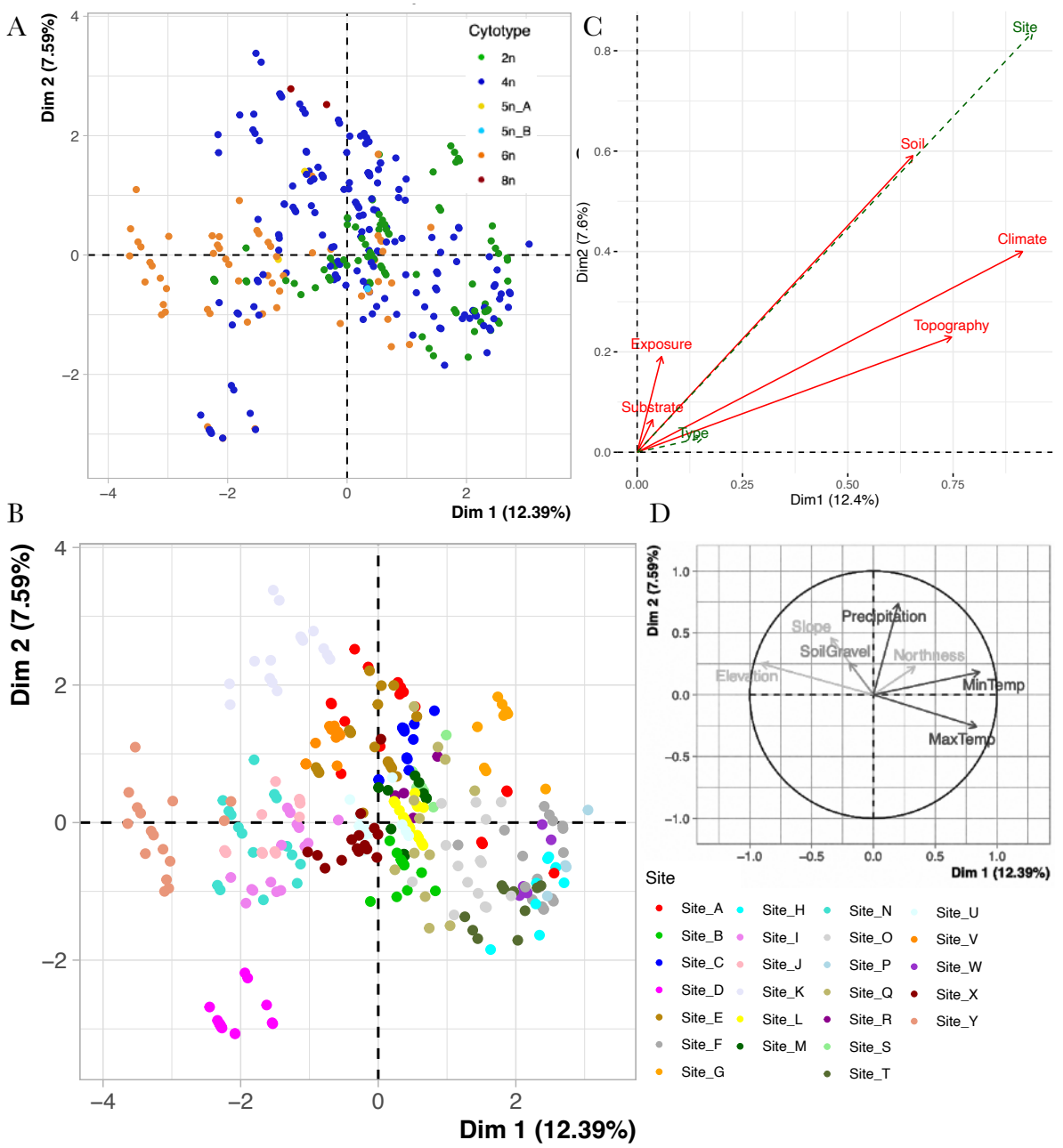

Figure 1.4: A- Multiple factor analysis (MFA) based on 10 abiotic environmental variables as predictors of cytotype distribution of *Oxalis obliquifolia* across Gauteng Province, with dimensions 1 and 2 only accounting for a cumulative 19.98% of the variation observed, colours grouped by cytotype. **B-** The same MFA plot with colours grouped by site. **C-** Contribution of each group of active (red) and supplementary (green) variables, in the construction of the first and second dimensions of the MFA. **D-** Correlation circle showing the 7 continuous variables used in the construction of the MFA.

(Table 1.2), which showed that although abiotic differences between both cytotypes and sites were statistically significant, site as the response variable had a far greater sum of squares value (21.858 vs 3.169), indicating that it accounted for the vast majority of the variation observed.

Table 1.2: Results of PerMANOVA analysis of abiotic variables associated with *Oxalis obliquifolia* cytotypes and collection sites

	Degrees of freedom	Sum of Squares	R 2	F statistic	P-value
Cytotype	5	3.169	0.09840	27.0309	**$1.0x10^{-3}$ ***
Site	24	21.858	0.67869	38.8416	**$1.0x10^{-3}$ ***
Cytotype:Site	16	0.590	0.01833	1.5735	**$1.0x10^{-3}$ ***
Residual	281	6.589	0.20458		
Total	326	32.206	1.00000		

*** indicates significant p-values**

1.4. Discussion

This investigation presents a detailed look at local ploidy variation in a plant species known to have a very large geographic range, larger than any other southern African *Oxalis*. Despite only a small portion of the overall distribution being included in this research, a surprising number of cytotypes were found in this species. Even more surprising, was the degree to which these different cytotypes co-occur.

Chromosome number in *Oxalis obliquifolia*

Genome size, chromosome number and ploidy level are fundamental genomic variables of plant taxa, and are of great importance when it comes to understanding species evolution and intraspecific diversity. Previous studies concerning the karyology of the genus *Oxalis*, have demonstrated a large degree of variation in chromosome number across different lineages and species, with a range of base chromosome numbers, including x = 5, 6, 7, 8, 9,11, 12, 14 and 17 (Moura *et al.*, 2020), with the majority having a base number of x = 7. Additionally, these chromosomes have been observed to exhibit a range of diverse morphologies with

regards to centromere placement, including metacentric, submetacentric, telocentric and acrocentric (de Azkue, 2000; de Azkue and Martinez, 1983; 1984; 1988; 1990).

For the first time, a base chromosome number of $x = 7$ is provided for the species *Oxalis obliquifolia*, with mostly metacentric to submetacentric morphology. This is consistent with what was expected based on published chromosome counts for closely related taxa, including *O. obtusa*, which likewise has been found to possess a base chromosome number of $x = 7$ (Krejčíková *et al.*, 2013*b*). Additionally, chromosomes were largely observed to be metacentric to submetacentric. Among some of the challenges posed in obtaining these counts for the *O. obliquifolia* included the very early stage of development of the inflorescence that was required in order to obtain pollen mother cells at just the right stage of meiosis. This meant that immature buds needed to be harvested, for dissection and anther isolation, when they were 1 mm, or less, in length. Additionally the chromosomes of *O. obliquifolia* were observed to be very small, and often difficult to clearly visualise, even under high magnification (at 1000x magnification). These data contribute to the growing body of karyological knowledge for the genus *Oxalis* (Heitz, 1927; Marks, 1956; Mathew, 1958; Sharma and Chatterji, 1960; de Azkue, and Martinez, 1990; Dreyer and Johnson, 2000; de Azkue, 2000; Sato *et al.*, 2008; Krejčíková *et al.*, 2013*b*; Vaio *et al.*, 2013; Moura *et al.*, 2020).

Intraspecific ploidy variation in *Oxalis obliquifolia*

The detailed approach to sampling employed in this investigation (10-15 individuals per locality) for 25 different sites across an area of roughly 9500 km^2, has shown that local populations of *O. obliquifolia* harbour an impressive amount of cytotype diversity. The cytotype diversity found is comparable to that encountered across the entire distribution of a closely related species, *O. obtusa* (Krejčíková *et al.*, 2013*a*; 2013*b*). Five distinct cytotypes (2x, 4x, 5x, 6x and 8x) and one possible case of aneuploidy (5x+) were encountered across the study area. This is exceptionally high, even when compared with cytotype distributions of other species at larger geographical scales (for example in Marhold *et al.*, 2010; Frajman *et al.*, 2015). The very close estimates of relative genome sizes for diploids, tetraploids, hexaploids and octoploids, suggest that these higher-level cytotypes are most likely autopolyploid in origin, which supports previous suggestions of very limited-to-no hybridisation events in the southern African *Oxalis* lineage (Salter, 1944). Although less likely, it is also possible that such close estimates for relative genome size may indicate possible hybridisation of species with

very close genome sizes, such as hybridisation events in polyploid complexes of other species (for example, in the genus *Sorbus*; Pellicer *et al.*, 2012).

Geographic distribution of cytotypes in Gauteng

This investigation has revealed a very complex pattern of cytotype distribution of *O. obliquifolia* across Gauteng, and a remarkable degree of sympatry (more than half of sampled sites) across different cytotypes. This high degree of co-occurrence made it difficult to discern any distinct patterns of cytotype distribution, an observation supported by the MFA results, which were unable to separate cytotypes based on abiotic variables. It is possible that more distinct patterns of cytotype distribution may be observed across larger parts of the distribution range (as seen in other studies such as: Hijmans *et al.*, 2007; Manzaneda *et al.*, 2012; Sutherland and Galloway, 2018; Semple *et al.*, 2021), as this would allow for larger variation and gradients in abiotic variables to be assessed. Indeed many studies have also shown that whole genome duplication has been directly linked to range expansion (for example in McIntyre, 2012; Voss *et al.*, 2012; Maguilla *et al.*, 2021), and can even result in increased invasiveness (te Beest *et al.*, 2012).

However, as demonstrated in this study, extensive sampling (more than just 3 or 4 individuals per site) is necessary to get an accurate idea of cytotype variation at individual sites, and thus the presence of mixed-cytotype populations may be underrepresented in existing studies that only have limited numbers of samples per site. It should also be noted that there are instances where studies on ecological (often climatic) differentiation between polyploids and diploids, have not always supported habitat segregation (for example in Godsoe *et al.*, 2013; Glennon *et al.*, 2014). Additionally, it is also possible that the resolution of abiotic variables used to assess cytotype distribution in this investigation, was at a scale that was too coarse to detect more subtle, or fine-grained differences in habitat (as noted by Kirchheimer *et al.*, 2016). It may be the case that if finer-scale data (perhaps at the level of individual accessions, or at a resolution of tens of meters) for abiotic variables were used, it would allow for the identification of micro-site level variability, which could potentially be correlated to cytotype.

While there was no definite patterns observed in the geographic distribution of cytotypes of *O. obliquifolia* in this system, there are some general trends that can be discerned, such as the higher frequency of diploids in the northern to western regions of Gauteng, and hexaploids occurring in higher numbers throughout regions in the south-east. These observations were

supported by statistically significant p-values for differences in abiotic variables associated with different cytotypes and sites. Tetraploid individuals were relatively evenly distributed across the entire study area. Overall, eight different cytotype combinations were encountered, three involving only the three majority cytotypes (i.e. 2x + 4x, 4x + 6x, 2x + 4x + 6x) and five more cytotype combinations that include at least one minority cytotype (i.e. 2x + 4x + 8x, 4x + 5x + 6x, 4x + 5x + $5x^{+}$, 4x + 8x, 6x + 8x). The occurrence of mixed-cytotype populations is well documented in many species (for example, in Burton and Husband, 1999; Weiss *et al.*, 2002; Španiel *et al.*, 2008) and have often been observed to indicate contact-zones between broader distinct distributions of single ploidy populations (such as those observed in, Husband and Schemske, 1998; Mráz *et al.*, 2012; Sabara *et al.*, 2013; Zozomová-Lihová *et al.*, 2015; Castro *et al.*, 2012). It remains to be seen whether this is the case in *O. obliquifolia* - future work should focus on a larger study area to reveal any discernible cytogeographic patterns. However, the high degree of cytotype co-occurrence (up to three at a single locality) suggest a highly complex system, where abiotic variables are not the primary drivers of observed cytotype distribution patterns.

1.5. Conclusion

Local populations of *Oxalis obliquifolia* harbour an exceptional amount of cytotype variation across a relatively small part of its overall distribution range. These findings support suggestions of higher polyploid incidence in the genus *Oxalis*, and that polyploid incidence in the sub-Saharan African region may be higher than previously thought. The remarkably high degree of sympatry in this system provides a unique and promising opportunity to investigate cytotype interactions and factors influencing cytotype distributions. Broad scale habitat segregation between diploids and polyploids was not observed, however further research across a larger portion of the distribution range of *O. obliquifoloia*, and taking into account more microclimatic variables as cytotype predictors, is crucial to determine whether the high degree of sympatry is a local phenomenon, or part of a broader pattern of the cytogeography of this species.

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CHAPTER 2: Morphological and phenological effects of polyploidy in *Oxalis obliquifolia*

2.1. Introduction

Polyploidisation events can have considerable consequences for factors that govern phenotypic expression. The instantaneous doubling, or multiplication, of DNA content following polyploidisation can result in substantial changes in epigenetic and transcriptomic regulation of gene expression (Schranz and Osborn, 2004; Adams and Wendel, 2005; Parisod *et al.*, 2010; Gao *et al.*, 2016), although the degree to which this occurs in some autopolyploids is not always clear, with observations in some synthetic autopolyploids demonstrating fewer than expected changes to gene expression (Martelotto *et al.*, 2005; Albertin *et al.*, 2005). Despite this, it is generally acknowledged that possessing multiple gene copies has the potential to result in lasting consequences for gene expression, and thus morphological, physiological, and phenological effects, giving rise to the manifestation of novel phenotypes (Wendel, 2000; Ramsey and Schemske, 2002; Bennett and Leitch, 2005; Comai, 2005; Balao *et al.*, 2011; Weiss-Schneeweiss *et al.*, 2013; Bigl *et al.*, 2019).

Gene expression can be described as either dosage-dependent or dosage independent (Feng *et al.*, 2020). There are a number examples where phenotypic expression is governed by genes that exhibit an allele-dosage dependency effect, and thus are directly impacted by polyploidisation events (Osborn *et al.,* 2003; Shi *et al.*, 2015), since the expression levels of dosage-dependent genes correlate with the number of copies of those genes (Osborn *et al.,* 2003). It has previously been suggested that the majority (two-thirds; Shi *et al.*, 2015) of alleles are subject to dosage-dependency effects, and that these genes are closely related to essential processes such as cell division, photosynthesis and metabolic functions (Shi *et al.*, 2015; Feng *et al.*, 2020). Dosage-independant genes (Shi *et al.*, 2015) have instead been associated with response to abiotic and biotic stress factors (Feng *et al.*, 2020). This creates ample opportunity for increased phenotypic variation (through multiple copies of dosage-dependent and dosage independent alleles on which selection can act) across different cytotypes (Bennett and Leitch, 2005; Chen, 2007). This topic has been the subject of numerous investigations since the beginning of the 20th century (Gates, 1909; Winge, 1917; Stebbins, 1947; DeMaggio and Stetler, 1971; DeMaggio and Lambrukos, 1974; Guo *et al.*, 1996; Balao *et al.,* 2011; Shi *et al.*, 2015; Tan *et al.*, 2016; Van Hieu, 2019). Investigations into the link between polyploidisation

and subsequent changes to phenotypic expression is important for understanding the evolution and ecological role of polyploids (Müntzing, 1936; Otto and Whitton 2000; Paterson, 2005; Otto, 2007; Flagel and Wendel, 2010; Van de Peer, 2017) in species populations.

This phenotypic variation among polyploids is one of the primary reasons that so many crop species are cultivated as polyploid varieties (Eigsti, 1957; Sattler *et al.*, 2016). Higher-ploidy crops often exhibit an increase in growth rate and/or size (Sattler *et al.*, 2016), as well as other variations that make polyploids more suitable for agriculture, such as their ability to better respond to abiotic (Stebbins, 1971; Ramsey and Schemske, 2002; Liu *et al.*, 2011) and biotic stresses, such as their improved resistance to pathogens (Oswald and Nuismer, 2007; Mehlferber, *et al.*, 2022), and their resistance and susceptibility to herbivory (Edger *et al.*, 2015; Hull-Sanders *et al.*, 2009; Segraves and Anneberg, 2016), and ability to cope with a lack of pollinator availability (through increase selfing ability; Stebbins, 1950; Hedrick, 1987). Furthermore, the study of artificial neopolyploids has demonstrated that polyploidisation has instantaneous and pronounced morphological, anatomical and physiological consequences (for example in Stanys *et al.*, 2006; Gaikwad *et al.*, 2009; Baker *et al.*, 2017; Wei *et al.*, 2019).

Polyploidy, and its associated physiological changes, have been linked to broader shifts in ecological tolerances and niche occupancy (Felber-Girard *et al.*, 1996; Levin, 2002; Baack, 2004; Adams and Wendel, 2005; Sonnleitner *et al.*, 2010). Some of these physiological changes can include changes in transpiration rates, water balance, hormone levels (Levin, 1983; 2002; Warner and Edwards, 1993), chlorophyll content (Dong *et al.*, 2017; Greer *et al.*, 2018), and response to abiotic stress, for example higher tolerance to increased salinity stress in *Robinia* L. polyploids (Wang *et al.*, 2013). The higher tolerance to environmental stresses may offer polyploids a competitive advantage over their diploid parents in circumstances where they co-occur. In particular, polyploids have been observed to have higher photosynthetic capacity (Coate *et al.*, 2012; Chen *et al.*, 2021) than diploids, thereby resulting in increased growth rates and more vigorous plants.

Changes in growth rates and photosynthetic capacity are often associated with changes in other anatomical and morphological features, including the thickness of the leaves (Sun *et al.*, 2015), the size of stomata (Speckmann *et al.*, 1965; Laere *et al.*, 2011; Marinho *et al.*, 2014; Zhang *et al.*, 2017), and the composition of photosynthetic pigments (Liu *et al.*, 2018). With regards to the effects of polyploidisation on morphology there have been extensive studies

and reviews on the topic (see Knight *et al*., 2005; Doyle and Coate, 2019). Previous studies have shown that polyploids tend to display changes in the size or number of particular cell types, including changes in the length of guard cells, epidermal cell area, and changes in the density of stomata (Beaulieu *et al*., 2008). Polyploidisation can also alter the size and shape of entire plant organs, for example alterations in the over-all size of shoots and leaf dimensions/ shape (Sugiyama, 2005; Trojack-Goluch and Skomra, 2013; Lan *et al*., 2020; Trojack-Goluch *et al*., 2021), changes in the size and shape of flowers and floral parts (Segraves and Thompson, 1999; Anssour *et al*., 2009; Nghiem *et al*., 2011; Trojack-Goluch and Skomra, 2013), and changes in seed size (Anssour *et al*., 2009; Chan *et al*., 2022).

The most well-known effect of polyploidisation is the "Gigas" effect (Randolph, 1941; Stebbins, 1971; Levin, 2002; Knight and Beaulieu, 2008; Sattler *et al*., 2016; Becker *et al*., 2022). First described by Gates (1909) it was named after the plant species *Oenothera lamarckiana* mut. *gigas*, and refers to polyploid size increase as a result of nucleotypic effects (Doyle and Coate, 2019), in other words the size effect derived from the increase in genomic DNA content on nuclei, independent of the effect of genes (Bennett, 1971; 1987; Levin, 2002). This nuclear size increase results in a cascading effect, whereby the size of individual cells also increase in response (Bennett, 1987; Balao *et al*., 2011; Snodgrass *et al*., 2017), and also manifesting at other higher organisational levels (Ramsey and Schemske, 2002).

The positive correlation between nuclear DNA content and cell size (Otto and Whitton, 2000; Doyle and Coate, 2019) has often been measured based on the size of stomatal guard cells or individual pollen grains (Masterson, 1994; Funamoto *et al*., 2006; Beaulieu *et al*., 2008; Marinho *et al*., 2014; Becker *et al*., 2022). Furthermore, if individual cells are found to be larger in higher ploidy-level cytotypes, measurements relating to cell density are likely to be negatively correlated with polyploidy (Levin, 2002; Chen *et al*., 2009). At higher organisational levels, such as at the tissue level, quantitative changes like decreased stomatal density (del Pozo and Ramirez-Parra, 2014; Monda *et al*., 2016; Robinson *et al*., 2018; Doyle and Coate, 2019) or hairs have been reported (Sosa and Dematteis, 2014; Chansler *et al*., 2016), in conjunction with larger cells and tissues. Ultimately, polyploidisation has generally been shown to result in a larger organ structures (including flowers, fruits, and leaves; Tang *et al*., 2010; Feng *et al*., 2017), or even in the size of the whole individual (Balao *et al*., 2011; Sosa *et al*., 2012; Hodálová *et al*., 2015).

Although, polyploidy does not always result in larger individuals, since increased cell size can conversely result in the decreased occurrence or rate of cell divisions (Noggle, 1946; Stebbins, 1971) in polyploids, and may in fact result in phenotypes that have more compact growth forms (Horn, 2002; Liu *et al.*, 2007; Blasco *et al.*, 2015; Sattler *et al.*, 2016; Hias *et al.*, 2017). However, it has generally been observed that polyploids most often do tend to exhibit larger organ structures and increased size (Porturas *et al.*, 2019). There is another consequence to increase cell size in polyploids, which involved cell division. Larger cells result in an increase in the time it takes for cell division (Bennett 1987, Francis *et al*. 2008), which can have the knock-on effect of decreasing growth rates (Levin, 2002; Otto, 2007; Maherali *et al.*, 2009). It has also been previously been suggested that this change in growth rate, as a result of prolonged divisions of larger cells, has been associated with changes in plant phenology (Noggle, 1946; Stebbins, 1971).

Many polyploids have been observed to exhibit distinct differences in phenology in comparison to their diploid parents (Stebbins, 1971; Segraves and Thompson, 1999; Pires *et al.*, 2004), and very often this manifests a direct consequence of slower growth rates, resulting in prolonged or delayed biological events, such as germination times (Keeble, 1912). One of the more common instances of this change in phenology relates to changes in flowering phenology (for example in Schranz and Osborn, 2000; Petit *et al.*, 1997). In particular, some studies have shown that polyploids exhibit prolonged (Bose and Choudhury, 1962) or delayed (Smith, 1946; Garbutt and Bazzaz, 1983) flowering, as was expected in the case of decrease growth rates. However, it has also been observed that changes in phenology, particularly flowering, do not always follow this pattern, and instead polyploid flowers may occur earlier than diploid flowers (Segraves and Thompson, 1999). It has been suggested that in such cases natural selection is the driver that effects phenological differences, after polyploidisation has occurred (Nuismer and Cunningham, 2005). Additionally, changes in phenology, in particular shifts to earlier flowering, have been associated with competition avoidance behaviour (Levin, 2009; Wolkovich and Cleland, 2010), and can even promote invasiveness in some species (te Beest *et al.*, 2012).

The effects of polyploidisation of phenotypic variation, including physiological, morphological and phenological changes, makes polyploidy an important factor in determining the interactions of polyploids with their biotic and abiotic environments, and have the potential to directly impact polyploid competition with their diploid parents, or competition avoidance behaviours. For this reason, changes to polyploid phenotype can

provide a mechanism for ecological niche differentiation (Müntzing, 1936). It is therefore important that research into the effects of polyploidistaion on phenotype in neopolyploids are undertaken to better understand cytotype establishment and persistence.

In this study, the morphological and phenological effects of polyploidy in *Oxalis obliquifolia* were assessed utilising different cytotypes (including diploids, tetraploids and hexaploids identified using standard flow cytometric techniques; see Chapter 1) grown in the context of a common garden experiment. In particular, the following questions were investigated: (1) Are there morphological differences between different cytotypes of *Oxalis obliquifolia*? (2) Is polyploidy associated with changes in phenology in *Oxalis obliquifolia*?

2.2. Materials and Methods

Sample Collection and Common Garden

A total of 98 individuals were collected from 12 different sites (between 6 and 10 per site) across 4000 km^2 in Gauteng (from December 2020 to March 2021), and comprising all three major cytotypes (diploids from 4 sites, tetraploids from 10 sites and hexaploids from 2 sites, with a particular focus on individuals from 5 mixed ploidy sites). A common garden experiment was set up and cytotypes were determined using standard flow cytometry protocols (see protocol described in Chapter 1, 1.2 Methods) as described by Doležel *et al*. (2007). Plants were then potted into 13 cm diameter plastic pots containing a homogenised mixture of sand and potting soil, with each being planted at a depth of 5 cm below the soil surface. Plants were then allowed to acclimate and enter dormancy for a full season (over-winter, beginning from the end of March 2021, to the end of August 2021). Over the course of a full growing season (334 days in total, beginning 28 August 2021 and ending 28 July 2022) plants were watered every second day (beginning 28 August 2021), with exactly the same amount of water per pot (using a 100ml beaker, filled to the brim for consistency), and ending when each plant re-entered dormancy. Plants were grown outside, under full sun conditions and were shuffled/rotated once a week to minimise the effect of possible micro-climate variation on individual plants.

Morphological Traits

A total of 17 plant morphological traits were measured at two separate time intervals, at the same point in time (over the course of two consecutive days) at the peak of the growing season (end of January 2022; Appendix 2A), and again two months after the emergence of each individual (Appendix 2B), in order to account for the possible effect of age on individual morphology. In total, morphological data were obtained for 98 individuals (24 diploids, 55 tetraploids and 19 hexaploids), including two qualitative and 15 quantitative characters. Quantitative measurements of size-related characters were collected manually using callipers, and a ruler with an accuracy of 1 mm. These included both foliar (petiole length, middle-leaflet length, middle-leaflet width, lateral-leaflet length, lateral-leaflet width) and floral (flower diameter, petal length, petal width, sepal length, sepal width, bract length, peduncle length) traits. The first flower to open (from the date of measuring) and the largest, mature leaf was consistently used to collect measurement data. Additionally, other quantitative data captured included the bract position (measured from the base of the peduncle), number of leaves (excluding those that had already completely senesced and/or detached, and no longer photosynthetically active) and number of inflorescences (including those that had already formed fruit, and immature inflorescences that were clearly identifiable). Shape characters were recorded as ratios between size measurements of principle organs, which were then log transformed prior to analysis. These included the ratio of middle-leaflet length to middle-leaflet width, lateral-leaflet length to lateral-leaflet width, petal length to petal width, sepal length to sepal width, and flower diameter to flower length. The investigation also included qualitative traits, specifically flower colour and the colour of the abaxial surface of the leaf. These were assessed and categorised using printed colour charts for comparison (Appendix 2C) and performed under full sunlight conditions to minimise inconsistency.

Phenological Shifts

Phenological data were also captured from plants included in the common garden experiment, beginning from the date of first watering on 28th August 2021. Records of the timing of biological events were recorded, with each individual being inspected for the timing phenological events at the beginning of each day. Recorded phenological events included the date of emergence, date of first anthesis, date of last flower senescence, as well as the date of final senescence, here defined as when the last leaf turned yellow. The monitoring period

continued until final senescence of the last green leaf of the last individual on the 29th of July 2022. These dates were used to generate count data, for both vegetative phenology (Appendix 2D) and flowering phenology (Appendix 2E). Vegetative phenology included the number of days to plant emergence from date of first watering, and number of days to final senescence from date of first watering, and the number of days to final senescence from date of emergence. Flowering phenology included days to first anthesis from date of first watering, and days from first anthesis to final flower senescence. For flowering phenology, due to the addition of the trait date of last flower (defined as the last flower senescing with no more inflorescences developing) later in the study, flowering phenology was analysed only based on those plants for which a date of first anthesis and date of last flower were available (this included 9 diploids, 14 tetraploids and 8 hexaploids).

Statistical analysis:

All analyses were conducted using R version 4.2.0 (R Core Team, 2022). Traits were assessed for autocorrelation (Appendix 2F) using Pearson's correlation coefficient and the cor() function (R Core Team, 2022), and correlated traits were excluded from the univariate analyses, using a degree of correlation number of |0.7| as a cut-off for identifying strong correlations (excluded and retained characters are shown in Table 2.1). High levels or correlation were observed within two sets of traits (Table 2.1), most of which related to sizes of different structures measured on the same organ. Out of the nine pairs of correlated traits identified, two (middle leaflet length and petal width) were randomly selected to be retained for further analysis. To test for differences in morphological traits between cytotypes, univariate analyses (Appendix 2F) were conducted using the base R Generalised Linear Model (GLM; glm() function; R Core Team, 2022) function. Traits that were retained were randomly selected from correlated sets of traits. In order to select the optimal data transformation and distribution families (see Appendix 2G) for each GLM, traits were initially assessed based on the type of data. For continuous variables (such as size measurements), a Shapiro-Wilk test (using the function shapiro.test(); Royston, 1982) was used to assess the normality of the data. Additionally, the boxcox() function (Box and Cox, 1964; Venables and Ripley, 2002) was used to estimate values of Lambda, in order to identify possible data transformations required to normalise the data, in cases where there were severe violations of model assumptions. GLMs for these continuous traits were then constructed by comparing Q-Q plots of residuals, AIC values, and residual deviance values, for each combination of data

Table 2.1: Strong ($|P| \geq 0.7$) trait correlations, indicating traits retained and removed for the univariate analysis of morphological trait variation among cytotypes of *O. obliquifolia*. Since the results show two sets of correlated traits (first six pairs and second three pairs), all traits except two (Retained trait) were removed.

	Correlated traits		
Retained trait	**Trait 1**	**Trait 2**	**r**
Middle leaflet length	Middle leaflet width	Middle leaflet length	0.81
	Lateral leaflet length	Middle leaflet length	0.83
	Lateral leaflet width	Middle leaflet length	0.82
	Lateral leaflet length	Middle leaflet width	0.88
	Lateral leaflet width	Middle leaflet width	0.93
	Lateral leaflet width	Lateral leaflet length	0.89
Petal width	Petal length	Flower diameter	0.80
	Petal width	Flower diameter	0.77
	Petal length	Petal width	0.75

transformation and distribution family (Gaussian, inverse Gaussian and Gamma). For count data, negative-binomial distributions were used (instead of Poisson distributions) in order to accommodate over-dispersion of the data. Ratios were modelled using a quasi-Poisson distribution, and in some cases the data were log transformed, where these resulted improved model fit (see Appendix 2G). All p-values were adjusted using the Benjamini and Hochberg (1995) post-hoc correction method for multiple comparisons, which is seen as a more conservative approach to account for the False Discovery Rate (FDR; Benjamini and Hochberg, 1995), using the p.adjust() function (R Core Team, 2022). Additionally, in order to identify significant differences between each pairwise combination of the three cytotypes included, a Tukey post-hoc test was performed, using the glht() function (in the multcomp package; Hothorn *et al.*, 2008).

Multivariate statistics (Appendix 2H), in the form of a Factor Analysis of Mixed Data (FAMD) and a Linear Discriminant Analysis (LDA), were also used to assess the differences in traits between different cytotypes, specifically using the FactoMineR package (Lê *et al.*, 2008), and MASS package (Venables and Ripley, 2002). Ordinations were visualised using ggplot2 (Wickham, 2016). These ordinations allowed for the inclusion of all covariate traits, and to

assess the relationship between multiple traits as predictors of cytotype. The FAMD (using the res.FAMD() function; Appendix 2I; Lê *et al.*, 2008) was conducted in order to test if differences in foliar and floral traits (both quantitative and qualitative) were correlated with different cytotypes, with individual plant IDs (Accession) and cytotype as supplementary variables. However, the two qualitative variables included did not contribute substantially to explaining the variability observed. Subsequently, the quantitative traits were further analysed, using a LDA (using the lda() function; Venables and Ripley, 2002), for associations with cytotype. The LDA is a guided approach that allowed for the maximisation of existing variability in the quantitative data, based on pre-defined groupings (in this case, cytotype).

To assess the relationship between polyploidy and phenology, Generalised Linear Models (GLM; Appendix 2J) were used, utilising the the glm() function (R Core Team, 2022). The GLMs used Poisson distributions (for count data with number of days as the unit of measurement) and, as above, the Benjamini & Hochberg (1995) correction and Tukey post-hoc tests (Hothorn *et al.*, 2008) were used to adjust p-values for multiple comparisons.

2.3. Results

When comparing the two different sets of data (individuals measured at the peak of the growing season, and individuals measured after 2 months since emergence), there were subtle differences observed in the effects on the multivariate statistics (assessed using Principle Component Analyses; Appendix 2K). However, despite these slight differences, both sets of data yielded very similar results in term of which types of characters (size-related traits) were most useful in explaining the variability observed between cytotype clusters, and with regards to the degree of separation between clusters. This means that the difference between time of emergence of the individual plants did not have a large enough impact on the data to qualitatively change the overall results of the analysis of morphological traits. For this reason, the following results focussed only on the data set of measurements taken at 2 months from the emergence of each individual (i.e. captured at the same time since emergence).

Univariate analysis of morphological traits

Results from the GLM analyses showed that there were distinct associations between cytotype and 9 of the 16 morphological traits included in the assessment (Table 2.2), with the difference observed between diploid individuals and at least one of the higher-ploidy cytotypes being significant. Significant differences between cytotypes were detected for 4 of

Table 2.2: Cytotype morphological quantitative traits (unit), means (s.d.), and adjusted p-values of Generalised Linear Model analyses, using Benjamini and Hochberg corrections (2x-4x, 2x-6x), and Tukey post-hoc test results (2x-4x, 2x-6x, 4x-6x).

Trait	2x	4x	6x	Bonferroni adjusted p-value 2x-4x 2x-6x	Tukey post-hoc 2x-4x 2x-6x 4x-6x
Foliar traits					
1. Middle leaflet length (mm)	12.2 (2.3)	15.4 (2.9)	16.6 (2.6)	**$2.56x10^{-7}$ *** **$3.85x10^{-8}$ ***	**$<1.0x10^{-4}$ *** **$<1.0x10^{-4}$ *** $1.69x10^{-1}$
2. Petiole length (mm)	83.8 (24.9)	97.6 (21.1)	88.0 (24.6)	**$1.23x10^{-2}$ *** $4.76x10^{-1}$	**$1.89x10^{-2}$ *** $7.52x10^{-1}$ $2.22x10^{-1}$
3. Number of leaves	16.5 (8.3)	11.7 (3.7)	12.8 (4.1)	**$7.54x10^{-5}$ *** **$1.63x10^{-2}$ ***	**$<1.0x10^{-3}$ *** **$4.24x10^{-2}$ *** $6.48x10^{-1}$
4. Ratio middle leaflet width to length	1.33 (0.17)	1.45 (0.17)	1.41 (0.11)	**$8.52x10^{-3}$ *** $1.43x10^{-1}$	**$1.29x10^{-2}$ *** $2.99x10^{-1}$ $6.26x10^{-1}$
5. Ratio lateral leaflet width to length	1.26 (0.13)	1.25 (0.11)	1.28 (0.13)	$8.24x10^{-1}$ $6.88x10^{-1}$	$9.73x10^{-1}$ $7.35x10^{-1}$ $5.32x10^{-1}$
Floral traits					
6. Petal width (mm)	7.9 (1.1)	10.2 (1.7)	11.2 (1.5)	**$4.12x10^{-9}$ *** **$3.57x10^{-10}$ ***	**$<1.0x10^{-3}$ *** **$<1.0x10^{-3}$ *** $6.60x10^{-2}$
7. Sepal length (mm)	5.5 (0.9)	6.4 (1.1)	6.6 (0.6)	**$2.01x10^{-4}$ *** **$2.01x10^{-4}$ ***	**$2.24x10^{-4}$ *** **$3.39x10^{-4}$ *** $6.78x10^{-1}$
8. Sepal width (mm)	2.3 (0.5)	2.5 (0.5)	2.8 (0.5)	$7.85x10^{-2}$ **$7.54x10^{-3}$ ***	$1.75x10^{-1}$ **$1.11x10^{-2}$ *** $2.14x10^{-1}$
9. Bract length (mm)	4.8 (1.6)	5.2 (1.9)	5.6 (1.2)	$5.40x10^{-1}$ $1.36x10^{-1}$	$8.10x10^{-1}$ $2.00x10^{-1}$ $3.35x10^{-1}$
10. Peduncle length (mm)	89.7 (21.7)	103.9 (20.1)	107.9 (24.3)	**$4.24x10^{-3}$ *** **$4.24x10^{-3}$ ***	**$6.33x10^{-3}$ *** **$9.30x10^{-3}$ *** $8.27x10^{-1}$
11. Ratio flower diameter to petal length	1.16 (0.16)	1.13 (0.09)	1.22 (0.23)	$3.74x10^{-1}$ $3.56x10^{-1}$	$6.43x10^{-1}$ $4.57x10^{-1}$ $6.92x10^{-2}$
12. Ratio petal length to width	1.91 (0.26)	1.85 (0.21)	1.75 (0.22)	$2.95x10^{-1}$ **$3.67x10^{-2}$ ***	$5.40x10^{-1}$ $5.66x10^{-2}$ $2.14x10^{-1}$

13. Ratio sepal length to width	2.42 (0.52)	2.58 (0.51)	2.41 (0.45)	$2.61x10^{-1}$ $8.72x10^{-1}$	$3.53x10^{-1}$ $9.85x10^{-1}$ $5.27x10^{-1}$
14. Ratio peduncle length to bract position	1.09 (0.04)	1.12 (0.11)	1.14 (0.07)	$5.26x10^{-2}$ $5.26x10^{-2}$	$1.05x10^{-1}$ $1.20x10^{-1}$ $9.16x10^{-1}$
15. Number of inflorescences	8.6 (4.8)	5.5 (2.9)	6.5 (3.0)	**$4.60x10^{-5}$ *** **$3.34x10^{-2}$ ***	**$<1.0x10^{-3}$ *** $8.33x10^{-2}$ $3.96x10^{-1}$
16. Difference in peduncle and petiole length (mm)	5.9 (20.5)	6.3 (23.7)	19.9 (22.0)	$9.27x10^{-1}$ $1.37x10^{-1}$	$9.95x10^{-1}$ $1.05x10^{-1}$ $6.18x10^{-2}$

*** indicates significant p-values based on GLM results**

the 5 foliar traits, and 5 of the 11 floral traits. Many of the size-related traits (both foliar and floral) were significantly different between cytotypes (Table 2.2). Count data for the number of a particular organ type (both number of leaves and inflorescences) were also significantly different between cytotypes (Table 2.2). There were fewer significant differences between cytotypes with regards to shape-related characters between diploids and higher ploidy-level cytotypes, except for the shape of the middle leaflets, petals and the position of the bracts on the peduncle. Diploids had smaller leaflets and higher numbers of leaves, than both higher-ploidy cytotypes. For two foliar traits (petiole length and middle leaflet shape), diploids were significantly smaller than tetraploids, but not than hexaploids. With regards to floral characters, diploids had smaller petal widths, sepal lengths, sepal widths, and peduncle lengths. Additionally, diploids had more inflorescences than polyploids, and the position of the bract along the peduncle was also significantly different, with diploids having the bracts positioned nearer to the top of the peduncle. The shape of the petals were also significantly different between diploids and hexaploids, with hexaploids having a larger petal length to petal width ratio than diploids.

However, even in instances where traits were found to have significant differences between cytotypes, they were largely still over-lapping (Figure 2.1), resulting in no trait being identified as a truly reliable predictor of cytotype, even though the general trend was that larger cytotypes had larger leaves, larger flowers, as well as fewer leaves and inflorescences. It is also interesting to note that the number of leaves and inflorescences were generally inversely proportional to the size of foliar and floral traits, when comparing cytotypes. In other words, diploids had smaller leaves and flowers, but more of them, than polyploids.

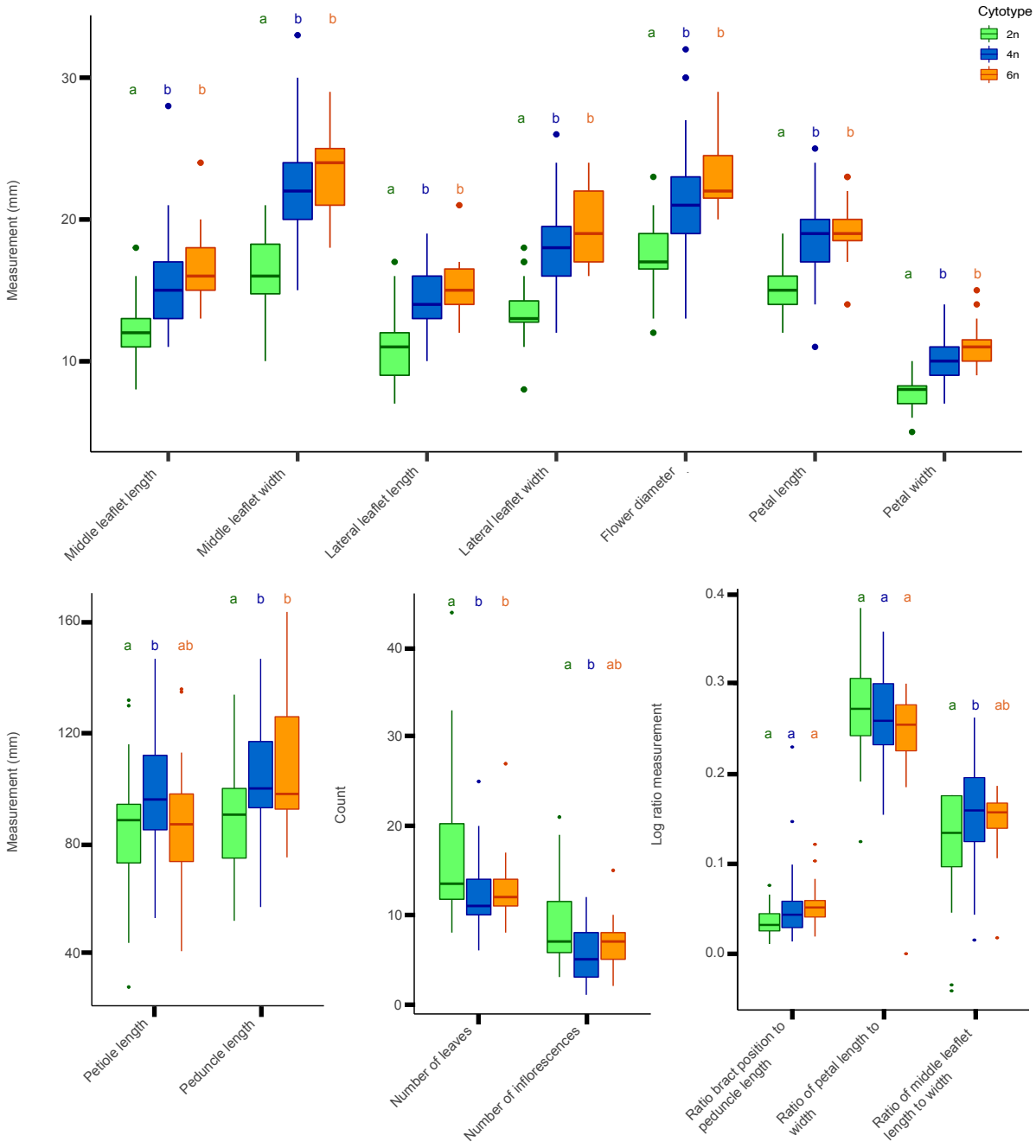

Figure 2.1: Box-plots of selected foliar and floral traits measured for different cytotypes (diploids - green; tetraploids - blue; and hexaploids - orange) included in a common garden experiment, and captured during the height of the growing season. Letters above plots denote statistically significant differences in values of traits associated with different cytotypes, based on GLM results and Tukey post-hoc test.

Morphological multivariate analysis

The results of the LDA (Figure 2.2), in which cytotypes were included as predefined groups, the percentage separation of cytotypes that is achieved by the first linear discriminant (LD1) axis, is 88.26%, and the percentage separation of cytotypes that is achieved by the second (LD2) linear discriminant axis, is 11.74%. In other words, based on the morphological data and variables used to construct LD1, 88.26% of the variation is accounted for and can be directionally applied to identifying a particular individual as belonging to a particular defined group (in this case cytotype). This LDA model had a prediction accuracy of 72.22%. This means that 72.22% of the time, LD1 and LD2 could be used to classify an individual into the correct cytotype. Based on the coefficients of linear discriminants the predictor variables that are most influential in creating the decision rule of the LDA model (in other word those that contribute most to creating LD 1), include lateral leaflet width, lateral leaflet length and petal width. Sepal length, petal length and sepal width were the most informative traits in constructing LD2. The multivariate analysis are suggests that size-related traits (both floral and foliar) are most informative in distinguishing between cytotype clusters.

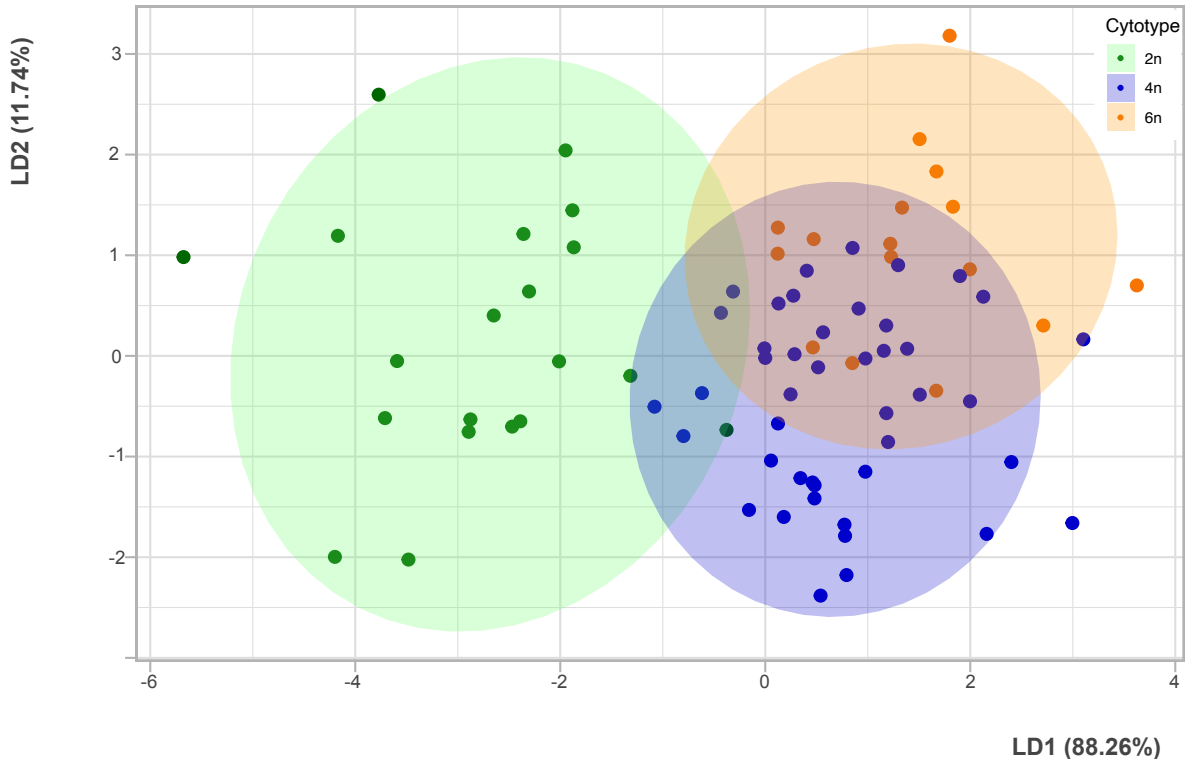

Figure 2.2: Linear discriminant analysis (LDA) constructed using all 23 quantitative morphological traits as predictors of cytotype (diploid - green, tetraploid - blue, hexaploid - orange), showing clear separation of clusters, with 95% confidence ellipses indicated for each group.

Phenological analysis

Significant phenological differences were observed between different cytotypes (Table 2.3 and Figure 2.3). Diploids emerged earlier (an average of 66.7 days after initial watering; Table 2.3; Figure 2.3A and 2.3B) than tetraploids (71.0 days average) and hexaploids (79.3 days average). Additionally, diploids had a longer growing season (average of 226.3 days), with a longer period spent above ground (prior to final senescence), than tetraploids (216.3 days) and hexaploids (200.8 days; Table 2.3; Figure 2.3A and 2.3B). Although between-cytotype differences in vegetative growth were slight, differences in flowering phenology were clearly more distinct than differences in other phenological variables based on the GLM analyses. Diploids begin flowering slightly earlier (113.7 days from first watering; Table 2.3; Figure 2.3C and 2.3D) than tetraploids (126.6 days from first watering) and hexaploids (130.5 days

Table 2.3: Phenological data (in days), means (s.d.), and adjusted p-values of Generalised Linear Model analyses, using Benjamini and Hochberg corrections (2x-4x, 2x-6x), and Tukey post-hoc test results (2x-4x, 2x-6x, 4x-6x).

Phenological Trait	2x	4x	6x	Bonferroni adjusted p-value 2x-4x 2x-6x	Tukey post-hoc 2x-4x 2x-6x 4x-6x
Vegetative Phenology n = 105					
1. Days to emergence (from date of first watering)	66.7 (29.1)	71.0 (31.3)	79.3 (29.0)	**2.68×10^{-2} *** **5.80×10^{-7} ***	6.78×10^{-2} **$<1.0\times10^{-4}$ *** **$<1.0\times10^{-4}$ ***
2. Days to final senescence (from date of first watering)	292.3 (26.7)	286.6 (40.2)	279.4 (38.8)	1.57×10^{-1} **1.46×10^{-2} ***	3.31×10^{-1} **2.58×10^{-2} *** 2.15×10^{-1}
3.Time above ground (from emergence to senescence)	226.3 (40.0)	216.3 (48.9)	200.8 (51.6)	**4.17×10^{-3} *** **4.45×10^{-9} ***	**1.13×10^{-2} *** **$<1.0\times10^{-4}$ *** **$<1.0\times10^{-4}$ ***
Flower Phenology n = 31					
4. Days to first anthesis (from date of first watering)	113.7 (29.3)	126.6 (33.8)	130.5 (28.5)	**1.79×10^{-8} *** **1.65×10^{-15} ***	**$<1.0\times10^{-4}$ *** **$<1.0\times10^{-4}$ *** **1.11×10^{-3} ***
5. Duration of flowering (first anthesis to last flower)	137.4 (28.0)	102.9 (31.2)	116.4 (22.1)	**1.14×10^{-13} *** **1.25×10^{-4} ***	**$<1.0\times10^{-4}$ *** **$<1.0\times10^{-4}$ *** **9.20×10^{-3} ***

*** indicates significant p-values based on GLM results**

from first watering). The largest difference was observed in the duration of the flowering period - diploids had a much longer flowering period (137.4 days; Figure 2.3C and 2.3D), on average, than either tetraploid (102.9 days) or hexaploid (116.4 days) plants. It is interesting to note that the polyploids (both tetraploids and hexaploids) exhibited a narrower peak flowering season that occurred towards the beginning of the longer diploid flowering season (Figure 2.3C and 2.3D).

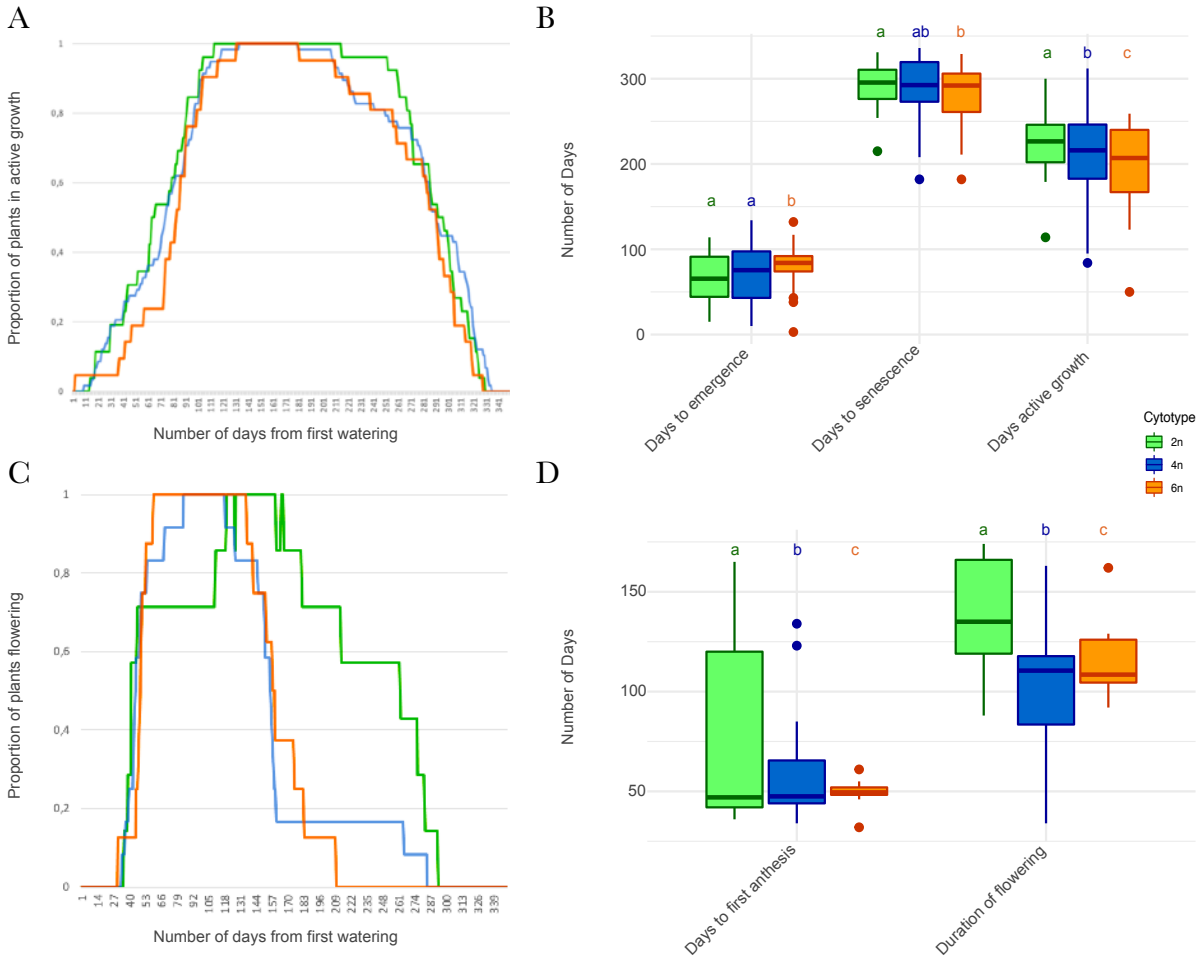

Figure 2.3: A- Proportion of actively growing *O. obliquifolia* individuals, including diploids (green), tetraploids (blue) and hexaploids (orange) in a common garden. **B-** Box-plots of plant vegetative growth phenology measured for different cytotypes (diploids - green; tetraploids - blue; and hexaploids - orange) included in a common garden experiment. **C-** Proportion of flowering *O. obliquifolia* individuals, including diploids (green), tetraploids (blue) and hexaploids (orange) in a common garden. **D-** Box-plots of flower phenology measured for different cytotypes (diploids - green; tetraploids - blue; and hexaploids - orange) included in a common garden experiment. Letters above plots denote statistically significant differences in values of traits associated with different cytotypes, based on GLM results and Tukey post-hoc test.

2.4. Discussion

The Gigas Effect in *Oxalis obliquifolia*

The results of this investigation showed a distinct association between the size of foliar and floral structures in *Oxalis obliquifolia,* and the increase in genome size, a clear indication of the Gigas effect. It has previously been reported that there is some degree of evidence for the occurrence of the Gigas effect in polyploids in the genus *Oxalis,* which are shown to have larger stomata, pollen and epidermal cells on average (Becker *et al.*, 2022), although the overall difference in size is comparatively small, than other examples of genera known to exhibit the Gigas effect (see Stebbins, 1971; Soltis *et al.*, 2014; Otto and Whitton, 2000, Porturas *et al.*, 2019), and this pattern is observed to be surprisingly inconsistent across *Oxalis* species (Becker *et al.*, 2022). However, here it is worth reiterating that for almost all size-related traits in this study, *Oxalis obliquifolia* polyploids showed size differences between cytotypes that were consistent with what was expected in the context of a system under the Gigas effect. In particular, the change in the average size of leaves and flowers between diploids and tetraploids were observed to be larger than 20%, which is consistent with the findings of Porturas *et al.* (2019), although in this example the size increase is shown to be consistent across the whole plant body (Porturas *et al.,* 2019).

Experimentally induced or synthetic polyploids are especially useful for understanding the effects of polyploidy on phenotypic variation (Sas-Nowosielska and Bernas, 2016), since the resulting individuals are considered to be free of the effects of "long adaptation", or are uninfluenced by selection (Hegarty *et al.*, 2013). Early research suggests that ploidy induced phenotypic variation in traits that are functionally related show strong correlations (Conner and Via, 1993; Balao, *et al.*, 2011), an idea known as "phenotypic integration" (Berg, 1960). Similarly, in this investigation, the effect of polyploidy on foliar and floral traits in *O. obliquifolia* were largely correlated within particular organs (ie. generally the morphological effect was consistent across the entire organ structure). This reinforces the idea of traits being organised into sets of interacting features, sometimes referred to as "modules" (Vasseur, 2022), which can generally be seen to be independent of one another (Wagner *et al.*, 2007; Klingenberg, 2008; Murren, 2012; Diggle, 2014).

However, some traits may become "decoupled" from one another over time (Balao *et al.*, 2011). This may be a result of physiological constraints (Vasseur, 2022), or trait divergence

driven by natural selection (Nuismer and Cunningham, 2005). One possible example of trait divergence in *O. obliquifolia* involves the bracts. While the entire reproductive structure (including the flower and peduncle) was significantly larger in polyploids than diploids, this was not the case with bract length. This may be due to the fact that the bracts are relict organs (plant parts that are largely free of selective pressure, and have lost their original function) on an otherwise highly modified structure with an essential, and highly specialised, function (that of facilitating sexual reproduction).

The increased size of *O. obliquifolia* polyploids may have important consequences for physiological processes, such as changes in efficiency of gaseous exchange, carbon fixation and water relations (Levin, 1983; 2002; Warner and Edwards, 1993; Vasseur, 2022), thereby impacting plant growth rates and plant vigour. This may have direct consequences for the ability of polyploids to respond to abiotic conditions/stresses (see Chapter 1), as well as their competitive ability (Van de Peer, 2021). Additionally, ploidy induced changes in the size of particular organs may impact resource partitioning in the plant, as illustrated in this study, where an increase in the size of a particular organ type (larger leaves or flowers) was also associated with the production of fewer numbers of those structures (fewer leaves or inflorescences).

In the case of trait divergence due to natural selection, natural selection can differentially mask the initial phenotypic effects of polyploidisation in some traits over time, while other altered traits remain unchanged, or are even enhanced, in response to selective pressures. This phenotypic variation is seen as an important aspect in allowing polyploids to potentially better adapt and exploit new ecological niches (Otto and Whitton, 2000). For example, polyploidy is observed to result in larger, or differently shaped, flowers (Garbutt and Bazzaz, 1983; Balao *et al.*, 2011), which may impact pollinator interactions (Taylor and Smith, 1980; Segraves and Thompson, 1999). Considering this, it is possible that polyploidisation in *O. obliquifolia* may be a relatively recent event, since the expected increase in size due to the Gigas effect is very much still evident in extant natural populations, and thus has not yet been obscured over generations that have been acted upon by natural selection. Conversely, it is also possible that selection may have favoured, and thus preserved the Gigas effect in this system.

It remains to be seen how variable these morphological traits are *in situ* (phenotypic plasticity; see Hahn *et al.*, 2012; Sánchez Vilas and Pannell, 2017). This experiment was conducted under controlled conditions, but in a field setting where environmental conditions can vary

over a small scale, trait differentiation between different cytotypes may be less pronounced, if trait expression is also influenced by environmental factors. Alternatively, it may also be the case that competition in the wild may also result in differences in morphology. This would be an interesting topic for further investigation. Generally, it is expected that more environmental variability would result in larger variation in phenotypic traits of different cytotypes, and a higher degree of overlap in those traits.

Phenological shifts

Diploids emerged above-ground slightly earlier and had longer active growth periods than polyploids. They also displayed an earlier onset of flowering and a much longer flowering season than both tetraploids and hexaploids. Both tetraploids and hexaploids had peak flowering times that were concentrated towards the beginning of the diploid flowering season. This is contrary to other studies where polyploids show distinctly prolonged (Bose and Choudhury, 1962) or delayed flowering times (Smith, 1946; Garbutt and Bazzaz, 1983), possibly as a results of slower growth rates associated with the Gigas effect. Shifts in flower phenology enable polyploids to escape direct competition with diploids for resources such as light and pollinators (Levin, 2009; Wolkovich and Cleland, 2010). In particular phenological shifts towards earlier flowering, as potentially evinced in *O. obliquifolia*, can potentially promote invasiveness in some polyploid species (Petit *et al.*, 1997; Pyšek *et al.*, 2009; te Beest *et al.*, 2012).

A number of other studies show distinct variation in flowering phenology between adjacent diploid and tetraploid populations in wind-pollinated species, (for example, Borrill and Linder, 1971; Lumaret and Barrientos, 1990; Van Dijk, 1991). It is believed that this serves as a primary mechanism to maintain reproductive isolation, driven by previous environmental disturbances (Stam, 1983), resulting in nonrandom migration of genes associated with the control of flowering time. However, in the case of *O. obliquifolia*, cytotypes co-occur, which suggests that another driver for the shift in flowering phenology exists in this system, unless reproductive isolation is present despite the high degree of cytotype sympatry (see Chapter 3). Differences in flowering time can exist in systems with sympatric polyploids and diploids (Clark, 1975; Lumaret and Barrientos, 1990; Petit *et al.*, 1997). Although there is evidence of an earlier beginning in polyploids in *O. obliquifolia*, the flowering period between polyploids and diploids still overlap substantially. Selection and trait differentiation through pollinator

interactions, may provide the answer to directional shifts in traits associated with reproduction.

Another mechanism that can result in differentiation in flowering time involves pollinator interactions. Since polyploidisation can result in changes to floral structure it may also have an impact on plant-pollinator interactions (Muchhala and Potts, 2007; Gómez *et al.*, 2014; Casazza *et al.*, 2017). For example, an increase in flower size, as observed in *O. obliquifolia*, may directly impact pollinator attraction with changes in flower shape or larger petals (Balao *et al.*, 2011; Tunbridge *et al.*, 2011; Casazza *et al.*, 2017) making polyploids more prominent and noticeable than diploids in mixed-ploidy populations. Polyploidy may also impact the availability of nectar to pollinators, either by altering the amount produced, or though ease of access due to altered flower morphology (Tunbridge *et al.*, 2011; Balao *et al.*, 2011). Such changes, in addition to phenological shifts, may encourage assortative mating within cytotypes (Husband and Sabara, 2004; Kennedy *et al.*, 2006) in the context of mixed-ploidy populations, such as in *O. obliquifolia* (Chapter 1). For example, the insect-pollinated *Heuchera grossulariifolia* Rydb. exhibits differences in both flower morphology and phenology, and the combination of changes to these floral traits may have resulted in the development of reproductive isolation (Segraves and Thompson, 1999) between diploids and polyploids. The change in flower size, coupled with the earlier shift in flowering period, may have similar effects in the insect-pollinated *O. obliquifolia,* thus reducing competition for pollinators between diploids and polyploids. However, it is still unclear whether this is achieved by polyploids attracting different types of pollinators, or if the same pollinators are involved, but that they preferentially visit larger polyploid flowers that occur towards the beginning of the flowering season.

2.5. Conclusion

There are clear indications that the Gigas effect is present in *Oxalis obliquifolia*, and this offers the opportunity for studying the impact of morphological differences between cytotypes in mixed-ploidy populations. Polyploids (tetraploids and hexaploids) tend to be larger than diploids (for both vegetative and reproductive traits) and this may have profound consequences for plant physiological processes, response to abiotic environmental conditions, and the competitive ability of polyploids. Additionally, the combination of larger flowers and potential shifts in flowering phenology may suggest possible pollinator interactions as a key

factor in facilitating assortative mating and competition avoidance behaviour, thus potentially enabling polyploid persistence in mixed-ploidy populations. This study raises a number of questions regarding the impacts of ploidy-induced phenotypic variation on different aspects of plant ecology, specifically with regard to biotic interactions, and how this may influence cytotype occurrence and polyploid success.

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CHAPTER 3: The frequency of polyploidisation and cytotype reproductive isolation in *Oxalis obliquifolia*

3.1. Introduction

Polyploidisation is a prime mechanism of sympatric speciation (Briggs and Walter, 1997; Otto and Whitton, 2000; Husband and Sabara, 2004; Sonnleitner *et al.*, 2013; Futuyma and Kirkpatrick, 2017), defined as "speciation […] in the absence of geographical barriers" (Lawrence, 2011; Otto and Whitton, 2000), and additionally is believed to be a possible driver for instantaneous speciation events (Ramsey and Schemske, 1998; Otto, 2007). This is ascribed to the fact that in a single generation, the hybridisation of two species and subsequent chromosome doubling (in the case of allopolyploids), or the fusion of unreduced gametes from same species parents (in autopolyploids), can result in the development of instant barriers to reproduction between polyploid offspring and their diploid parents (Ramsey and Schemske, 1998). Although historically autopolyploids have been less often accepted as separate species than allopolyploids, despite often being morphologically distinct and, importantly, reproductively isolated from their diploid parents (Soltis *et al.*, 2007), autopolyploids may in fact be more important for species diversification and evolution than what was previously assumed (Soltis *et al.*, 2007; Otto, 2007).

Reproductive isolation between polyploids and their diploid parents has been the topic of much research concerning population dynamics and the evolution of polyploid species complexes (Segraves and Anneberg, 2016). Many studies have investigated reproductive isolation among different cytotypes in autopolyploid populations (Thompson and Lumaret, 1992; Petit *et al.*, 1999; Hardy *et al.*, 2001; Husband *et al.*, 2002; Baack, 2005; Baldwin and Husband, 2010; Castro *et al.*, 2012; Koutecký *et al.*, 2012; Sonnleitner *et al.*, 2013), and the extent of reproductive isolation between diploids and polyploids has been shown to be a major factor in determining population structure, and patterns of cytotype distribution, in mixed-ploidy populations or cytotype contact zones (Koutecký *et al.*, 2012; Sonnleitner *et al.*, 2013).

Reproductive isolation is a consequence of different prezygotic and postzygotic barriers that exist among different taxa (see Ramsey *et al.*, 2003; Lowry *et al.*, 2008; Christie *et al.*, 2022) and cytotypes (Husband and Schemske, 2000; Husband and Sabara, 2004; Rieseberg and

Willis, 2007; Widmer *et al.,* 2009; Köhler *et al.*, 2010; Roccaforte *et al.*, 2015; Van de Peer *et al.*, 2021). It also includes he potential for complex interactions between these barriers. Some prezygotic barriers rely on ecological niche differentiation (Felber-Girard *et al.*, 1996; Husband and Schemske, 2000; Baack and Stanton, 2005; te Beest *et al.*, 2012; Sonnleitner *et al.*, 2013) via range shifts and the establishment of geographic isolation, differences in flowering phenology (Levin, 1983; Petit *et al.*, 1999; Husband and Schemske, 2000; Levin, 2002), or changes to pollinator interactions (Segraves and Thompson, 1999; Thompson *et al.*, 2004; Kennedy *et al.*, 2006; Laport *et al.*, 2021). Shifts in pollinator preferences or changes in pollinator assemblage, may manifest as a result of changes to flower morphology, flower colour, the amount of nectar produced, and even changes in the scent of polyploid flowers (Husband and Sabara, 2004; Jersáková *et al.*, 2010; Gross and Schiestl, 2015; McCarthy *et al.*, 2015).

Differentiated pollinator interactions results in assortative mating among polyploids and diploids, where mating patterns in a population are non-random between individuals (Rodríguez, 1996; Lawrence, 2011). In the polyploid context, this would mean that mating between same ploidy individuals is more likely to occur than between polyploids and diploids. The result of this intracytotype mating between polyploids would be a polyploid lineage, distinct from diploids, and potentially leading to complete reproductive isolation, even in sympatry (Segraves and Thompson, 1999; Anssour *et al.*, 2009; Balao *et al.*, 2011). However, even in instances where pollination between diploids and polyploids does occur, other reproductive barriers can still prevent the production of viable offspring, which include the effects of polyploidy on chromosomal rearrangements and recombination, complications arising in chromosome segregation during cell divisions, and other genetic incompatibilities that interfere with zygote and/or endosperm formation (Williams *et al.*, 1999; Husband *et al.*, 2002).

Postzygotic barriers, reproductive barriers subsequent to successful zygote formation, are another important aspect of reproductive isolation and can be generally classified as either intrinsic or extrinsic barriers (Coyne and Orr, 2004; Sutherland and Galloway, 2021). Intrinsic reproductive isolating barriers refer to the innate inability of the F1 offspring to reproduce (offspring inviability or sterility; Dobzhansky, 1937; Sutherland and Galloway, 2021). Extrinsic barriers can be viewed as those subsequent barriers to reproduction, where the F1 offspring have the potential to produce viable offspring of its own, but secondary factors (such as ecological and/or behavioural attributes, like differences in reproductive

phenology and pollinator segregation) prevent this from occurring (Coyne and Orr, 2004; Sutherland and Galloway, 2021). Perhaps the most well known and extensively studied postzygotic barrier to reproduction between polyploids and diploids is known as the "triploid block" (Ramsey and Schemske, 1998; Petit *et al.*, 1999; Köhler *et al.*, 2010), where interploid crosses between tetraploids and their diploid parents can potentially result in triploid offspring that are often inviable, due to a malfunctions in endosperm and zygotic development (Köhler *et al.*, 2010).

This strong degree of reproductive isolation between diploids and polyploids is an important aspect that contributes to minority cytotype exclusion (Levin, 1975; Felber, 1991; Husband, 2000). Essentially, under a system involving a triploid block, newly emergent polyploids would have no compatible reproductive partners available, and are thus limited in their ability to successfully establish in existing diploid populations (see Introduction Chapter). However, this is not always the case, and in some circumstances the fate of newly emergent tetraploids may in fact be reliant on the rate of triploid formation, where a triploid bridge (Husband, 2004; Köhler *et al.*, 2010; Mason and Pires, 2015; Schinkel *et al.*, 2017) can facilitate the production of higher ploidy-level cytotypes, and where reproductive isolation between polyploids and diploids is incomplete or absent. This means that the degree of triploid fitness (in terms of both ability to reproduce and ability to survive; Stebbins, 1950) relative to parent diploids and tetraploids, and the ploidy-level and rate of functional gametes produced by triploids (Husband, 2004; Suda and Herben, 2013), will determine if more polyploids will enter the system through interploid crosses, thus impacting the rate of polyploid formation.

The rate of polyploidisation is higher in many lineages than what was originally expected (Soltis and Soltis, 1999), and the frequency of polyploidisation events can vary substantially, suggesting different predispositions for polyploidisation and polyploid establishment/ persistence between different lineages (Ramsey and Schemske, 1998; Husband *et al.*, 2013). Some of the factors that promote increased rates of polyploidisation in populations include the rate at which unreduced gametes are formed (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 2002; Ramsey, 2007) and the ability of polyploids to overcome the limitations imposed on them by minority cytotype exclusion.

With regards to the production of unreduced gametes, or 'gametic non-reduction' (Harlan and de Wet, 1975), since polyploids can form by the fusion of two unreduced ($2n$) gametes resulting in a tetraploid ($2n + 2n$, or odd ploidy-levels in the case of the fusion of reduced and unreduced gametes; Futuyma and Kirkpatrick, 2017), it could be expected that the rate of

polyploid formation is positively correlated to the frequency of unreduced gamete production in a given population (Soltis and Soltis, 1999). The rate at which unreduced gametes are formed may increase in natural diploid populations in response to environmental stress (Bretagnolle and Thompson, 1995; Mason *et al.*, 2011; Pécrix *et al.*, 2011; De Storme *et al.*, 2012; Sora *et al.*, 2016; Van de Peer *et al.*, 2021). Specific individuals may exhibit disproportionately elevated 2n gamete production, and thus could be major contributors to emergent polyploid populations (Soltis and Soltis, 1999). In particular, once a polyploid individual arises and begin to produce viable gametes, that automatically results in the increased frequency of unreduced gametes available in populations. In other words, once a polyploid individual emerges, it may facilitate and increase the rate of emergence for other polyploid individuals.

Alternatively, if new polyploids are unable to reproduce with diploids, or there is no triploid bridge, then polyploids must overcome minority cytotype exclusion, and expand their occurrence, through other mechanisms. Polyploidisation overcomes this challenge either by facilitating the breakdown of barriers to self-fertilisation (Rodriguez, 1996; Mable, 2004; Baack, 2005; Rausch and Morgan, 2005; Robertson *et al.*, 2011; Oswald and Nuismer, 2011; Fowler and Levin, 2016) in out-crossing species, or by increased clonal reproduction (Gustafsson, 1948; Stebbins, 1957; Husband *et al.*, 2013; Herben, *et al.*, 2017) and increased perenniality (Otto and Whitton, 2000; Rice *et al.*, 2019). These strategies would allow polyploids to persist in the landscape (Baack, 2005; Rausch and Morgan, 2005; McGrath and Lynch, 2012), increasing the chance of persistence until sexual reproduction becomes possible (i.e. until another compatible polyploid mate emerges).

In this study, the degree of reproductive isolation, and differences in seed set (as a potential indicator of fecundity) between different cytotypes (including diploids, tetraploids and hexaploids, identified using standard flow cytometric techniques; see Chapter 1) in *Oxalis obliquifolia* are assessed. Additionally, the rate of polyploidisation, as a possible factor in determining the high degree of sympatry in local populations, is also tested. In particular, the following questions were investigated: (1) Are different cytotypes of *Oxalis obliquifolia* reproductively isolated from one another? (2) Does polyploidy degrade barriers to self-fertilisation in *O. obliquifolia*? (3) Does maternal cytotype (diploid or polyploid) have an influence on seed set or the success rate of crosses? (4) And finally, are polyploidisation events frequently occurring in this system?

3.2. Materials and Methods

Crossing experiment

In order to determine the degree of reproductive isolation, and potential to produce hybrids, between the three major cytotypes including diploids, tetraploids and hexaploids; for cytotype identification procedure see Chapter 1, Materials and Methods) of *Oxalis obliquifolia*, artificial pollination experiments, conducted by hand and under controlled conditions, were performed with individuals (including 1140 crosses, of which 432 used maternal diploids, 499 used maternal tetraploids and 209 used maternal hexaploids collected from 12 different localities selected from across Gauteng Province, South Africa. This crossing experiment was designed to assess for the presence of potential barriers to seed production, based on the procedure described by du Preez *et al.* (2018). The pollination treatments involved in crosses between cytotypes were as follows: (a) self-pollination (flower stigma pollinated with an anther from the same flower) (b) within-cytotype pollination (flower stigma pollinated using an anther taken from a compatible flower morph, from an individual of the same cytotype), (c) between-cytotype pollination (flower stigma pollinated using an anther taken from a compatible flower morph, from an individual of a different cytotype). Manual pollinations were conducted from 7am to 12pm daily during the peak flowering period (from September 2020 to March 2021) using accessions of each cytotype, kept in open-air growing conditions. A fine pair of forceps (sterilised with alcohol) were used to collect and transfer anthers with pollen to compatible and unfertilised stigmas for each of the different crosses. As *O. obliquifolia* has a tristylous mating system, all crosses were conducted between compatible stigmas and anthers of the same level in plants with compatible stylar morphs (tall-, mid- and short-styled; du Preez *et al.*, 2018). Each floret was then emasculated (removal of all the remaining anthers) using alcohol sterilised forceps. Unwanted pollinator-vectored pollen was controlled for by the removal of petals and by covering the pollinated maternal flower with an empty teabag, tied at the based of the flower, which also helped to retain the seed after dehiscence of the fruit, since *Oxalis* seeds are typically explosively ejected from the capsule. All flowers that were unused on a particular day were removed so as to avoid confusing them with subsequent new virgin flowers. Unsuccessful fertilisation and seed set was measured by peduncles that withered and detached within two weeks (on average) of anthesis. For successful crosses, intact teabags were inspected each day for fruit dehiscence and seed release, and seeds from each cross were counted to determine the seed set for each cross.

DNA extraction, sequencing and analysis

To account for relatedness between individuals and test the possible number of polyploid origin events (either by independent polyploidisation or intercytotype hybridisation) represented in local *O. obliquifolia* populations, at least two representatives of each different cytotype (diploids, tetraploids and hexaploids) sampled from each site, with a particular focus on mixed ploidy sites, were studied using molecular techniques. Fresh leaf material from a total of 86 individuals was collected and placed in silica-gel for rapid drying and long-term storage. DNA extractions were based on the procedure described in Oberlander *et al.* (2004), using a modified 2X CTAB method of Doyle and Doyle (1987). First, silica-dried leaf tissue (approximately 0.4-0.6 g) was ground with liquid nitrogen, in a 70% alcohol-sterilised and thoroughly dried mortar and pestle. Subsequently, 500 µl of 2X CTAB extraction buffer (with 0.2% mercaptoethanol) was added to ground up tissue in a 1.5 ml Eppendorf and placed in a heating block at 60°C, for 45 minutes. Next, 500 µl of chloroform-isoamylalchohol (24:1 by volume) was added to each sample, and gently, but thoroughly, mixed for 10 minutes. Samples were then centrifuged for 5 minutes at 7000 x g. The upper aqueous phase was then removed using a wide-bore pipette, and dispensed into a new Eppendorf tube. A 2/3 volume of cold isopropanol (stored at -20°C) was added and mixed, before the sample was stored over-night, at -20°C, to facilitate nucleic acid precipitation. Samples were then centrifuged at low speed (3000 x g) for 2 minutes, and supernatant removed. Next, 1.5 ml of wash buffer (mixture of 40mM ammonium acetate solution and ethanol, in a 1:3 ratio, by volume) was added to the pellet and gently perturbed to resuspend it. After 20 minutes, during which the pellet in the wash buffer was gently swirled at regular intervals, the sample was again centrifuged at low speed (3000 x g) for 3 minutes. The supernatant was then removed, and the remaining DNA pellet was allowed to air dry, by placing the Eppendorf tube into a heating block, set at 30°C, for a minimum of 30 minutes, to evaporate off all remaining ethanol. The dry pellet was then redissolved in 200 µl of TE buffer, by gently swirling it and placing it in the fridge (set a 4°C) overnight. The quality and quantity of DNA yielded from the extractions were then assessed using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc., USA).

In order to assess the degree of intraspecific diversity among cytotypes of *O. obliquifolia*, four different genomic regions were selected for amplification and analysis, with the appropriate primers. These were: chloroplast intergenic regions trnH-psbA and trnS-trnG (Hamilton, 1999); nuclear ribosomal DNA (rDNA) internal transcribed spacer region (ITS; Sun *et al.*,

1994); and single-copy nuclear-encoded chloroplast-expressed glutamine synthetase (ncpGS; Oberlander *et al.*, 2010). The polymerase chain reactions (PCR) consisted of these reagents at the following concentrations: 12.5 µl of Ampliqon *Taq* MasterMix, 8 µl distilled water, 0.5 µl of 50 mMol $MgCl_2$, 1 µl of 10 µMol each primer, and 2 µl of template DNA, totalling approximately 25 µl. The PCR thermocycling protocols used for each primer pair were as follows:

- trnH-psbA: an initial denaturation step of 96°C for 5 min, followed by 35 cycles of denaturation/ annealing/extension at 96°C for 45 s, 53°C for 1 min, and 72°C for 30 s. A final extension step of 72°C for 5 min was included.
- trnS-trnG: an initial denaturation step of 96°C for 5 min, followed by 40 cycles of denaturation/ annealing/extension at 96°C for 45 s, 52°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 5 min was included.
- ITS: an initial denaturation step of 94°C for 3 min, followed by 35 cycles of denaturation/ annealing/extension at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. A final extension step of 72°C for 5 min was included.
- ncpGS: an initial denaturation step of 96°C for 5 min, followed by 35 cycles of denaturation/ annealing/extension at 96°C for 30 s, 52°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 7 min was included.

The success of the PCR amplifications were determined using standard agarose gel electrophoresis techniques. Successfully amplified PCR products were submitted for standard post-PCR clean-up, and dideoxy terminated Sanger sequencing, performed by the Central Analytical Facility at the University of Stellenbosch (http://www.sun.ac.za/english/research-innovation/caf). Chromas version 2.6.6 (www.technelysium.com.au) was used for chromatogram base calling verification, and BioEdit version 7.2.5 (Hall, 1999) was used for assembling of contigs and manual DNA alignment. Nucleotide polymorphisms reflecting potential intraspecific diversity were coded using standard IUPAC degenerative coding. Sequences were screened for potential contamination using BLAST searches and Genbank (NCBI) submissions. An initial set of samples (8 individuals, including 2 diploids, 4 tetraploids and 2 hexaploids from both the same mixed-ploidy sites and across different sites) were first amplified using all 4 primer pairs, and the resulting sequences were assessed for the presence of single nucleotide polymorphisms (SNPs), in order to determine the usefulness of each

marker, for the purpose of this study. The marker that yielded the highest number of SNPs between individuals was used for the remaining 78 accessions.

Statistical analyses

Unless otherwise indicated, analyses were conducted using R version 4.2.0 (R Core Team, 2022). The effect of different types of crosses (self-pollination, within-cytotype pollination, between-cytotype pollination), and the interaction with the cytotype of the maternal parent, was assessed using seed-set (the number of seeds resulting from a particular cross; Appendix 3A) as a proxy for the presence of possible prezygotic barriers to reproduction, as well as an indicator of potentially higher fitness between cytotypes. In order to assess the degree of barriers to successful seed formation, and accommodate the zero-inflated distribution of the seed set data, a hurdle model was used. This was done utilising the hurdle() function (Zeileis *et al.*, 2008; Appendix 3B) as part of the "pscl" package (Jackman, 2020). A negative binomial distribution was used for the seed-set Count data (seed-set above 0), and a binomial distribution for the Zero (success *vs.* failure to produce seed) count data. Additionally, in order to identify significant differences between each pairwise combination of the three types of crosses, and maternal cytotypes included, a Tukey post-hoc test was performed, using the emmeans() function (in the emmeans package; Lenth, 2022).

In order to test the hypothesis that same ploidy-level cytotypes, collected from different sites, are in fact more closely related to one another than to individuals of a different cytotype two methods were used. First, hierarchical clustering based on molecular distance/similarity and based on ITS sequences (Appendix 3C), was used to construct a dendrogram, to visualise the relatedness between individuals. A consensus tree with posterior probabilities was constructed using MrBayes software (parameters: nst = 6, rates = gamma; Ronquist *et al.*, 2012), through CIPRES online portal (Miller *et al.*, 2010), and visualised using FigTree version 1.4.4. Additionally, A parsimony tree with bootstrap support values was constructed using PAUP software (Swofford, 1991; also accessed through CIPRES), and included as a figure inset. Secondly, in order to statistically test this relatedness between individual haplotypes, an AMOVA (Analysis of molecular variance; Meirmans and Liu, 2018) was also included. In AMOVA, population structure was tested against cytotype and site. Statistical tests were conducted in the programme Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010); Appendix 3D).

3.3. Results

ITS was the most suitable marker for the assessment of intraspecific diversity in this system (having the largest number of single nucleotide polymorphisms), with all other markers included producing mostly uniform (ncpGS) or identical (trnH-psbA; trnS-trnG) sequence data (as is expected in the case of autopolyploidy). The ITS sequence alignment was 790 bp in length, with 15 sites found to be variable among the 82 individuals included. Within these variable sites, 13 were determined to be parsimoniously informative, with 2 singletons.

Success rate of crosses

In total 1140 crosses were performed (see Appendix 3E for summary data/data spread). These included all combinations of the interaction between the type of cross performed (self-pollinated, within-cytotype and between-cytotype) and the maternal cytotype (diploid, tetraploid or hexaploid; Figure 3.1). With regards to self-pollination success rates (calculated as a percentage of total crosses performed), diploid (1 successful *vs.* 101 unsuccessful) and hexaploid (1 successful *vs.* 77 unsuccessful) maternal cytotypes had similar results with

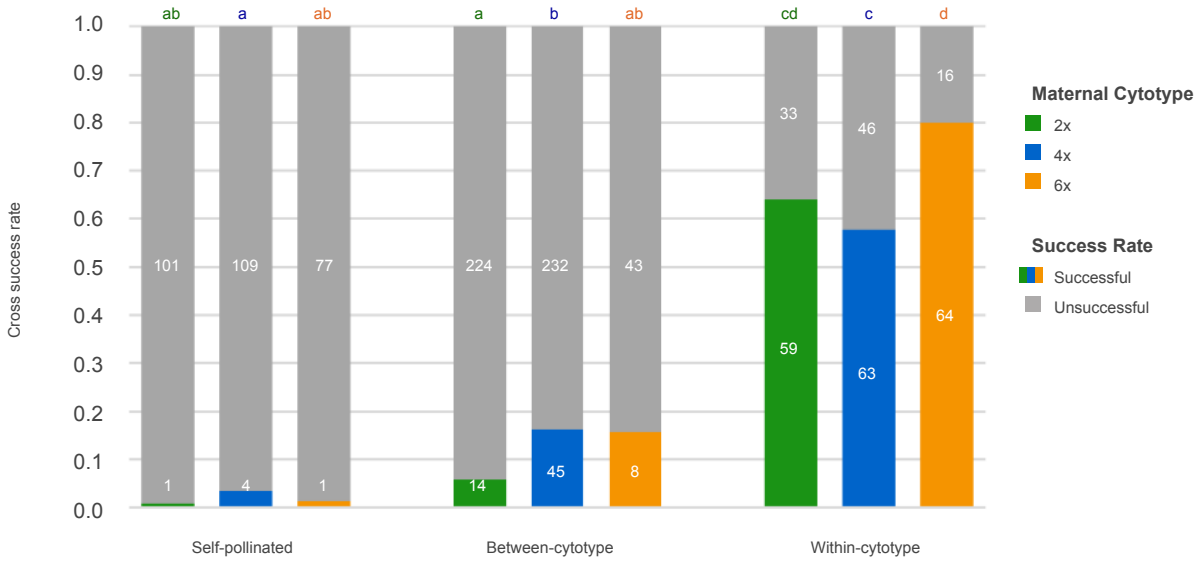

Figure 3.1: Proportion of successful (1 or more seeds) *vs.* unsuccessful (0 seeds) crosses for each combination of maternal cytotype (diploids - 2x - green; tetraploids - 4x - blue; hexaploids - 6x - orange) and type of cross (within cytotype; between cytotype; self-pollinated). Internal numbers indicate the count for successful or unsuccessful crosses for each type. Letters above plots denote statistically significant differences based on Tukey post-hoc test results.

Table 3.1: Hurdle model results of seed set as the response variable for the interaction between the type of cross (self-pollinated, within-cytotype or between-cytotype) and maternal cytotype (diploid(2x), tetraploid(4x) and hexaploid(6x)), indicating the back-transformed estimate, the back-transformed upper and lower 95% confidence intervals (CI), z value, and p-values.

	Estimate	Upper 95% CI Lower 95% CI	Z value	P-value
Zero hurdle model coefficients				
Intercept: Between Cytotype:2x	0.0588	0.0969 0.03514	-10.064	**< 2.0x10^{-16} ***
Self:2x	0.0098	0.00138 0.0663	-1.768	7.70x10^{-2}
Within Cytotype:2x	0.6413	0.7324 0.53867	9.557	**< 2.0x10^{-16} ***
Between Cytotype:4x	0.1625	0.2107 0.12354	3.539	**4.02x10^{-4} ***
Self:4x	0.0354	0.0905 0.01335	0.152	8.79x10^{-1}
Within Cytotype:4x	0.5780	0.6670 0.48360	-3.233	**1.23x10^{-3} ***
Between Cytotype:6x	0.1569	0.2835 0.08044	2.304	**2.12x10^{-2} ***
Self:6x	0.0128	0.0854 0.00180	-0.547	5.85x10^{-1}
Within Cytotype:6x	0.8000	0.8737 0.69814	-0.483	6.29x10^{-1}
Count hurdle model coefficients				
Intercept: Between Cytotype:2x	5.81407	8.8658 2.7624	6.580	**4.70x10^{-11} ***
Self:2x	1.08824	3.9822 -1.8057	-1.214	2.25x10^{-1}
Within Cytotype:2x	19.47950	24.1071 14.8519	4.128	**3.67x10^{-5} ***
Between Cytotype:4x	8.46729	10.8788 6.0558	1.240	2.15x10^{-1}
Self:4x	3.94611	7.9866 -0.0944	0.615	5.38x10^{-1}
Within Cytotype:4x	15.60558	19.2284 11.9828	-1.723	8.49x10^{-2}
Between Cytotype:6x	8.41479	14.0683 2.7613	0.853	3.94x10^{-1}
Self:6x	0.00007	0.0174 -0.0173	-0.080	9.37x10^{-1}
Within Cytotype:6x	13.31569	16.4080 10.2233	-1.612	1.07x10^{-1}

*** indicates significant p-values, and adjusted p-values, based on hurdle model results**

approximately 1.0% and 1.3% respectively, of self-pollinations resulting in the production of seed. This is compared with an approximately 3.5% success rate (4 successful *vs.* 109 unsuccessful) for self-pollinations of tetraploid maternal cytotypes. Overall there was no significant difference in the self-pollination success rates (Table 3.1; Figure 3.1) between different maternal cytotypes. In other words, barriers to selfing appear to be intact across all cytotypes. It is possible that the limited successful self-pollination crosses may be due to the presence of unwanted pollen (contamination) from another individual, despite measures taken to avoid this.

The self-pollination success rates provide a useful basis from which to assess the other types of crosses, and control for errors due to the presence of unwanted pollen and false successes of other types of crosses. The success rate of between-cytotype crosses involving diploids as the maternal cytotype (14 successful *vs.* 224 unsuccessful, or approximately 5.9%, and) was not significantly different from the success rate for the self-pollination crosses with maternal diploids (Figure 3.1). This was also the case for between-cytoype crosses with maternal hexaploids (8 successful *vs.* 43 unsuccessful; 15.7%). However, there were significant differences between self-pollination success rates and between-cytotype crosses with tetraploid (45 successful *vs.* 232 unsuccessful; 16.2%) maternal cytotypes. In other words, maternal tetraploids were able to successfully cross with other cytotypes and produce seed (Figure 3.1). As expected, within-cytotype cross success rates were substantially and significantly higher than self-pollination and between-cytotype cross success rates, across all maternal cytotypes. Diploids had a within-cytotype success rate of about 64.1% (59 successful *vs.* 33 unsuccessful). Tetraploids had a within-cytotype success rate of about 57.8% (63 successful *vs.* 46 unsuccessful), and hexaploids had the highest within-cytotype success rate of approximately 80.0% (64 successful *vs.* 16 unsuccessful).

Seed-set among successful crosses

Of those crosses that did produce seed, there was a significant difference between different types of crosses (Figure 3.2). In particular, seed-set was higher in between cytotype crosses than seed-set for successful self-pollinations, which is potentially to be expected in a species that is generally known to be self-incompatible. The highest number of seeds were produced in within-cytotype crosses, a pattern that was consistent across all maternal cytotypes (Figure 3.2). There was a significant difference between the number of seeds produced by between-

cytotype (mean of 5.5 seeds, min = 2.0, max = 13.0) and within-cytotype (mean of 19.0 seeds, min = 1.0, max = 48.0) crosses, with diploid maternal parents (Table 3.1; Figure 3.2). The only successful self-pollination of a maternal diploid plant produced two seeds.. In the case of the single successful self-pollination cross with a hexaploid maternal parent, 1 seed was produced. Out of the 4 successful self-pollination crosses with tetraploid maternal parents, a mean of 2.5 seeds (min = 1.0, max = 13.0) were produced. Self-pollination seed-set between all three maternal cytotypes were not significantly different from one another (Figure 3.2). The mean number of seeds produced by between-cytotype crosses with tetraploids as the maternal parent was 4.0 seeds (min = 1.0, max = 20.0), compared to the mean of 9.5 seeds (min = 2.0, max = 20.0) produced from between-cytotype crosses with hexaploids as the maternal parent. With regards to within-cytotype crosses with tetraploids and hexaploids as maternal parents, the mean seed-set for these crosses was 11.0 seeds (min = 1.0, max = 42.0) and 13.0 seeds (min = 1.0, max = 36.0), respectively. Finally, diploids produced the highest

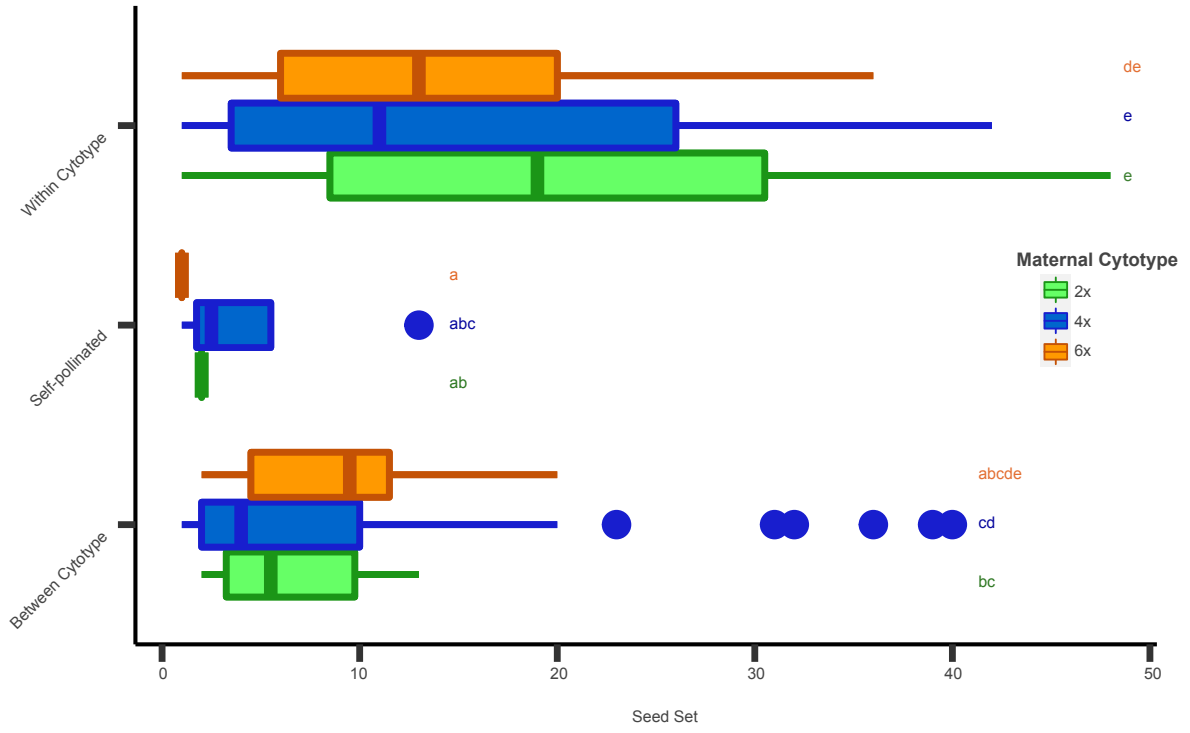

Figure 3.2: Box-plots showing the seed-set for successful crosses for each combination of maternal cytotype (diploids - green; tetraploids - blue; hexaploids - orange) and type of cross (within-cytotype, between-cytotype and self-pollinated). Letters alongside box-plots denote statistically significant differences based on Tukey post-hoc test results.

seed-set for within-cytotype crosses (mean = 19.0 seeds) compared with both tetraploids (mean = 11.0 seeds) and hexaploids (mean = 13.0 seeds), although this was not determined to be significantly different than the other within-cytotype crosses with tetraploids and hexaploids.

Gene flow and polyploidisation frequency

In dendrograms constructed from ITS data, diploids and polyploids form two discrete clusters (Figure 3.3), with both the parsimony tree and bayesian consensus tree giving very high support values (bootstrap value of 94, and posterior probability of 100, respectively) for the separation of these clusters. There is only one exception, of a single diploid individual (accession OF016), found at a mixed-ploidy site comprising both diploids and tetraploids, that was found to be more similar to the polyploids. Based on similarity, individuals cluster according to ploidy (diploids vs polyploid) and not according to site. Furthermore, while diploids are largely distinct from the polyploids (suggesting that they are reproductively isolated in the wild), within the polyploid cluster tetraploids and hexaploids are very much equally resolved within the same large polytomous branch, suggesting possible gene flow among polyploids.

Tests of population structure using AMOVA on ITS data followed two approaches. In the first instance, haplotypes were grouped by site, thereby allowing for the existence of distinct cytotype populations, within individual sites. In this scenario, the variation was largely explained by differences between cytotypes, within sites, rather than differences observed between sites. Furthermore, very little difference was observed within cytotypes, across individual sites. When haplotypes were grouped according to cytotype, almost all of the variation was observed between cytotypes, and not site, and these differences were again found to be significant. When looking at the sequences in more detail, within the diploid lineage 12 loci were found to be variable across the 790 bp length of sequence, with 6 sites being parsimoniously informative. This compared to the polyploid lineage, where only 4 sites were observe to be variable across tetraploids and hexaploids, with 3 sites being parsimoniously informative.

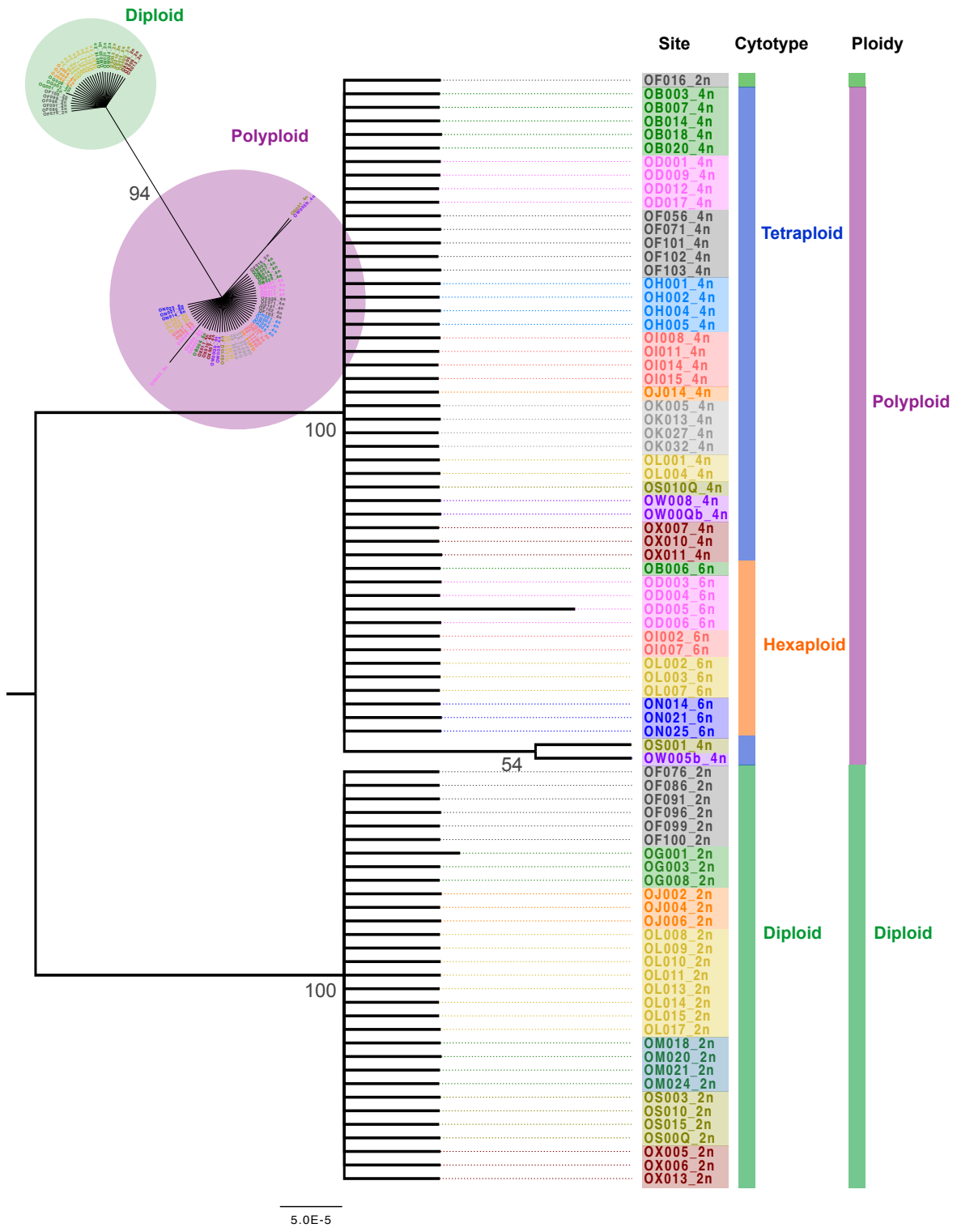

Figure 3.3: Bayesian consensus tree constructed using ITS sequence data, with branch lengths (branch lengths with support under 50 have been collapsed) and posterior probabilities, from *Oxalis obliquifolia* individuals of different cytotypes (diploids - green bars; tetraploids - blue bars; hexaploids - orange bars) collected from different sites (same sites indicated using coloured tree tips) across Gauteng. Cytotype grouping shown as vertical bars. **Figure inset:** presents the true unrooted tree with branch lengths, with bootstrap support indicated.

Table 3.2: Results of two AMOVA analyses conducted using ITS sequences of 82 individuals of *Oxalis obliquifolia* (including diploids, tetraploids and hexaploids), with grouping done based of ploidy and locality, and using a distance matrix constructed using the Kimura-2P model.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	P-value
AMOVA analysis 1: Grouped by site					
Among sites	13	58.255	-0.10664	-8.12	**<1.0x10^{-4} ***
Among cytotypes, within sites	9	38.310	1.31557	**100.21**	**<1.0x10^{-4} ***
Within cytotypes	59	6.127	0.10385	7.91	6.39x10^{-1}
Total	81	102.693	1.31279		
AMOVA analysis 2: Grouped by cytotype					
Among cytotypes	2	81.133	1.56464	**84.13**	**<1.0x10^{-4} ***
Among sites, within cytotypes	20	15.433	0.19131	10.29	**<1.0x10^{-4} ***
Within sites	59	6.127	0.10385	5.58	**<1.0x10^{-4} ***
Total	81	102.693	1.85980		

*** indicates significant p-values**

3.4. Discussion

This study provides a comprehensive assessment of the degree of reproductive isolation, and selfing ability, among different cytotypes of *Oxalis obliquifolia*, with evidence of strong, though not complete, barriers to hybridisation and gene flow between diploids and higher ploidy-level individuals. Furthermore, many shared haplotypes and the consequent lack of population structure between tetraploids and hexaploids suggests substantial gene flow between polyploid cytotypes, but not into diploids, which exist as their own distinct lineage.

Differences in seed-set

It has been well established that polyploidisation can result in decreased self-incompatibility in out-crossing species (Oswald and Nuismer, 2011; Fowler and Levin, 2016). Barriers to selfing serve to prevent inbreeding (Heizmann, 1992), and thereby promote genetic diversity in

species populations. However, a break-down in these barriers can facilitate reproductive success for minority cytotypes (by avoiding the need for available, compatible mates), thereby mitigating the challenges of minority cytotype exclusion. In this system, polyploidy was not associated with a break-down of self-incompatibility among higher-ploidy cytotypes of *Oxalis obliquifolia*. There was minimal seed-sed and success rates for the vast majority of self-pollinated crosses across all cytotypes. This is consistent with observations made in previous studies that have found that self-incompatibility can remain intact in polyploids (for example in Mable, 2004), contrary to expectation based on studies that have shown otherwise (such as, Husband and Schemske, 1997; Cook and Soltis, 2000).

The effects of polyploidy on seed-set can differ dramatically between different species. In *O. obliquifolia*, diploid within-cytotype crosses yielded the highest mean seed-set, but this was not determined to be significantly higher than that of polyploid within-cytotype crosses, similar to what was observed in studies by Münzbergová (2007) and Castro *et al.* (2011). However, other studies have shown that higher seed production by diploids is possible (for example in Burton and Husband, 2000; Münzbergová and Skuhrovec, 2017), which suggests a fitness advantage for diploids that would enable them to continue to coexist in mixed-ploidy populations, by virtue of their potentially higher levels of fecundity. In other instances polyploids may be capable of producing more seeds than diploids (Černá and Münzbergová, 2013; Gross and Schiestl, 2015), thus facilitating potentially rapid range expansion and persistence. It is also worth noting that in *O. obliquifolia* the seed-set associated with hexaploid within-cytotype crosses was significantly higher than tetraploid within-cytotype crosses. This may indicate a fitness advantage for hexaploid individuals that could facilitate hexaploid establishment among mixed tetraploid and hexaploid populations. It remains to be seen however, whether these reproductive dynamics would follow the same general patterns under different environmental conditions, since the current study area only focusses on a relative small part of the overall distribution of *O. obliquifolia*.

Between-cytotype crosses were generally not significantly different from the background selfing rate, for diploid and hexaploid maternal cytotypes in *O. obliquifolia*, there was some evidence to suggest that maternal tetraploid between-cytotype crosses did have a higher success rate than tetraploid self-pollinations. This was further supported by evidence of higher seed-set for between-cytotype crosses with maternal tetraploids. This supports the idea that between-cytotype crosses are possible, but to a lesser extent between diploids and polyploids, and thus may potentially yield viable seed (although seed viability and germination did not

form part of this investigation; Burton and Husband, 2000). The noticeably low success rate, and seed-set associated with between-cytotype crosses with maternal diploids in *O. obliquifolia*, may offer evidence of strong barriers to reproduction between diploids and polyploids.

There is precedent for sympatric diploid and tetraploid populations demonstrating strong interploid reproductive isolation (Coyne and Orr, 2004; Husband and Sabara, 2004). One major obstacle to successful hybridisation between diploids and polyploids involves the triploid block (Husband and Sabara, 2004; Köhler *et al.*, 2010), or the production of inviable, sterile or low fitness triploid offspring, as a product of hybridisation between diploid and polyploid individuals. This is due to the fact that gametes produced by triploids are most often non-functional, as a result of aneuploidy and an imbalance in the number of chromosomes during meiosis (Satina and Blakeslee, 1937; Dujardin and Hanna, 1988; Hassan and Rehman, 2017). The triploid block is one possible explanation for the reproductive isolation observed between diploids and polyploids in *O. obliquifolia*. It is notable that no triploid individuals were encountered in the field (see Chapter 1 results), even at sites where diploids and tetraploids co-occurred. Polyploidisation can also reduce pollen viability (Ramsey and Schemske, 2002), which in turn can impact seed set (Galen and Gregory, 1989; Tiffin *et al.*, 2001), and even germination success (Ramsey and Schemske, 1998). This investigation showed that interploid crosses in *O. obliquifolia* yielded seed in an artificial context, and that there were clear differences in the number of seeds produced between different types of crosses and maternal cytotypes (with reduced seed set in polyploid within-cytotype crosses, which is in agreement with findings by Galen and Gregory, 1989). Tests of seed viability and germination would form a fruitful avenue for future research on polyploid fitness in this system. We could not test this as part of this study, as the germination cues for *O. obliquifolia* are unknown and no natural germination of harvested seed occurred during the study period.

Another possible explanation for the reproductive isolation observed in wild *O. obliquifolia* populations may involve pollinator-mediated reproductive isolation (Segraves and Thompson, 1999; Coyne and Orr, 2004) or assortative mating. There is evidence to suggest there are distinct differences in the size of flowers and flower phenology in *O. obliquifolia* (see Chapter 2), both of which are factors that could allow pollinators to differentiate between cytotypes (Segraves and Thompson, 1999; Husband and Sabara, 2004; Husband and Schemske, 2000) in sympatry, thereby strengthening assortative mating (Kennedy *et al.*, 2006).

Asymmetrical reproductive isolation

One of the major implications of this investigation was that in *Oxalis obliquifolia* the maternal cytotype involved in a particular type of cross was a significant factor in the success rate of between cytotype pollinations. This is consistent with recent work that suggests that reproductive isolation between higher ploidy-level cytotypes may be incomplete or less intact compared with barriers to reproduction between diploids and polyploids (Hersch-Green, 2012; Sonnleitner *et al.*, 2013; Hülber *et al.*, 2015; Sutherland and Galloway, 2021). This is also consistent with another study on *Campanula rotundifolia* polyploids (Sutherland and Galloway, 2017), which suggested that gene flow may be asymmetric when comparing diploid-tetraploid crosses and tetraploid-hexaploid crosses.

Evidence for this was not only demonstrated in the crosses performed under controlled and artificial conditions, but was also supported by ITS population structure, which suggested a clear distinction in the degree of gene flow between the polyploid and diploid lineages in wild populations of *O. obliquifolia* (as was also the case in Greiner and Oberprieler, 2012). However, ITS markers are subject to the effects of concerted evolution (Alvarez and Wendel, 2003), whereby the often multiple copies of this marker display high degrees of uniformity as a result of different sequence homogenisation processes (Alvarez and Wendel, 2003), which ultimately can mask original ITS haplotypes via introgression. In the context of *O. obliquifolia*, this concerted evolution may ultimately have the effect of overwriting the signal of multiple polyploidisation events. However, this was deemed unlikely, given that it would require the polyploid ITS haplotype to consistently overwrite all other new polyploid haplotypes, in every case where a new polyploidisation events occurred.

The distinctly separate lineages of diploids and polyploids observed in this study suggests that the frequency of independent polyploidisation events (arising from diploid progenitors) are not occurring rapidly enough to explain the high degree of cytotype sympatry (see Chapter 1) observed in *O. obliquifolia*, at least within the study area. However, within the polyploid lineage the numerous shared haplotypes across cytotypes suggest possible hybridisation events, or possibly independent polyploidisation events, resulting in the rapid production of higher-ploidy level cytotypes. Among polyploids the fusion of reduced and unreduced gametes can generate cytotypes of higher ploidy-levels, or by successful reproduction between tetraploids of independent origin (Ramsey and Schemske, 1998). It is worth noting that the one diploid individual with a polyploid haplotype (accession OF016) was found at the same site as another polyploid individual (accession OF101), which displayed evidence in its sequence

data (Appendix 3F) of a second minority haplotype (a discernible background sequence) with clear similarities to that of the other diploids. Importantly, these individuals were found at a site where diploids and polyploids co-occur (see Chapter 1), and may potentially indicate a rare instance of *in situ* gene flow, between the polyploid lineage and the diploid lineage. However, since this was the only instance where this phenomenon was encountered, and it is unknown if crosses including these individuals among other diploids would result in viable offspring, it remains to be seen if there is more evidence to support this potential backward introgression of polyploid genetic material. It is also worth noting that this particular site (Faerie Glen Nature Reserve in Pretoria) was one of the more disturbed sites included in this study, which may have an impact on these findings. This may potentially be due to changes in environmental/ecological factors in this context (such as pollinator interactions, or abiotic stress) that could, for example, result in changes to the frequency of unreduced gamete production and patterns of intercytotype pollination events.

As found in this system, unidirectional gene flow amongst polyploids has also been observed in other polyploid complexes (Greiner and Oberprieler, 2012; Hülber *et al.*, 2015). If there is strong reproductive isolation between polyploids and their diploid parents, it may result in differences in diversification rates between the two distinct lineages, particularly when there is homogenising gene flow among higher ploidy-level cytotypes, which could result in lower diversification rates (Costa *et al.*, 2014; Sutherland and Galloway, 2017). Additionally, diploids and polyploids may develop secondary reinforcement to reproductive isolation (Husband and Sabara, 2004), through assortative mating or pollinator-mediated selection, while higher ploidy-level cytotypes engage in local hybridisation in sympatry. This implies that localities with sympatric tetraploids and hexaploids have the potential for increased between-cytotype gene flow when compared with those sites with co-occurring diploids and tetraploids. It is also worth noting, that the only pentaploid individuals encountered in the field (see Chapter 1 results), were encountered at a site with both tetraploid and hexaploid individuals. This may indicate a "pentaploid bridge" (Peskoller *et al.*, 2021; Šemberová *et al.*, 2021) involved in the production of higher ploidy-level cytotypes, and coincides with increased seed set for between-cytotype crosses involving tetraploids and hexaploids, than tetraploids and diploids.

Autopolyploids and species concepts

The above findings highlight a number of aspects of autopolyploid biology that directly relate to the ongoing debate of whether autopolyploids may be considered as different species to their diploid progenitors. Soltis *et al.* (2007) identified two major reasons why autopolyploids have not been recognised as distinct species, or afforded their own nomenclature under the current taxonomic system. The first reason is simply that traditionally different cytotypes have been subsumed under a single recognised species. This has been attributed to the fact that speciation by autopolyploidy was originally viewed as a rare occurrence (Stebbins, 1947). However, the current prevailing paradigm recognises autopolyploidy as a far more more widespread and important factor in land plant evolution. In fact, Soltis *et al.* (2007) further suggested that the previously assumed rarity of autopolyploidy may have been linked to the fact that taxonomists did not adequately recognise polyploids as distinct biological enities. The second reason for autopolyploids not being assigned their own name or classification concerns the long-standing tradition of employing phenetic or morphological species concepts (Soltis *et al.*, 2007), which is largely viewed as an out-dated approach in light of contemporary molecular techniques. However, it has long been recognised that species can be defined according to many different species concepts (Coyne and Orr, 2004), depending on the philosophical inclination of the taxonomist. Indeed, many autopolyploids meet the prerequisites to be recognised as distinct taxa under different species concepts (Soltis *et al.*, 2007), and it has further been suggested that autopolyploids may give rise to cryptic species (Parisod *et al.*, 2010; Eriksson *et al.*, 2017). In the case of *Oxalis obliquifolia*, it could be argued that it conforms to the requirements of the biological species concept (being largely reproductively isolated), potentially the diagnostic and phylogenetic concepts, and even arguably the morphological (based on size characters) species concepts, if the Gigas effect can be shown to consistently express in natural systems, and also wether these patterns are consistent across other parts of the species' geographic distribution, given that this investigation was limited to a relatively small area. However, it remains to be seen whether ecological distinctions would support this recognition. Furthermore, not recognising the polyploids as separate entities, in this system at least, would undercount the number of separate gene pools, with potential management and conservation implications as well.

3.5. Conclusion

Reproductive isolation between diploids and higher-ploidy level cytotypes is an important factor in determining polyploid establishment and success. In out-crossing species, such as *Oxalis obliquifolia*, this is especially important, as new polyploids are limited in their ability to reproduce in the absence of compatible mates. However, previous studies have shown that once one polyploid arises more are likely to follow, often due to the increase in the proportion of unreduced gametes in a system. This may also be due to interploid hybridisation, however it remains to be seen if seeds produced from interploid crosses in *O. obliquifolia* are able to germinate, reach reproductive maturity and produce viable offspring at levels capable of sustaining triploid bridges to the generation of new polyploids. This study has also revealed that the degree of reproductive isolation in an artificial setting and in the wild may be different. Given the high degree of sympatry, along with other morphological and phenological evidence, one possibility is that pollinators may play a substantial role in facilitating assortative mating, and reinforcing reproductive isolation between diploids and polyploids. Despite remarkable morphological similarity, remarkable sympatry, and marked potential gene flow, diploids and polyploids are behaving as if they exist as entirely separate biological entities. This raises the question of whether current practices of taxonomy and nomenclature are sufficient to adequately recognise the true diversity present among polyploid complexes, and consideration should be given as to how this may impact our assumptions of polyploidy in the context of plant evolution and speciation.

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GENERAL CONCLUSIONS

The purpose of this study was to investigate some of the primary factors governing polyploid establishment and persistence, specifically in the context of local populations of *Oxalis obliquifolia*. This topic was chosen to address some of the fundamental questions regarding polyploid success in the face of minority cytotype exclusion, and factors that contribute to patterns of cytogeography in polyploid complexes. Additionally this project contributes valuable data and findings on the occurrence of polyploidy in a widely distributed species in sub-Saharan Africa (outside of the Greater Cape Floristic Region), a region that has been generally lacking in studies that have focused on polyploidy, and its ecological and evolutionary significance.

This study has offered some unique insights into the patterns of cytogeography and cytotype diversity of a widespread grassland geophyte. For the first time, a chromosome count is provided for *O. obliquifolia*, and this investigation has revealed a substantial degree of cytotype diversity across a relatively small portion of the its overall distribution, which is comparable to that observed across other species entire distributions. This extraordinary degree of cytotype sympatry is very unlike those patterns of cytotype distribution observed in the Global North (where the majority of such studies have been conducted), where polyploid complexes have largely exhibited a pattern of distinctly separate cytotype distribution ranges, with varying degrees of overlap at contact zones. It is possible that the high degree of sympathy observed in this system may be part of a much larger contact zone, but more research across a much broader part of the distribution range would be required to verify this. This also immediately raises the question, why are these different cytotypes able to co-exist so successfully, and have not followed the expected pattern where one cytotype eventually excludes another, depending on their relative fitness? Future studies should focus on questions relating to how recently these polyploids have arisen, and also to what degree vegetative propagation of this species has enabled the persistence of higherploidy cytotypes, and impact distribution patterns in this polyploid complex.

Diploids and polyploids of *O. obliquifolia* display a remarkable degree of cytotype diversity and sympatry in local populations. Diploids and polyploids seem to share the same abiotic niche, however evidence provided in Chapter 3 shows that diploids and polyploids are effectively reproductively isolated from one another in the wild, despite interploid crosses resulting in

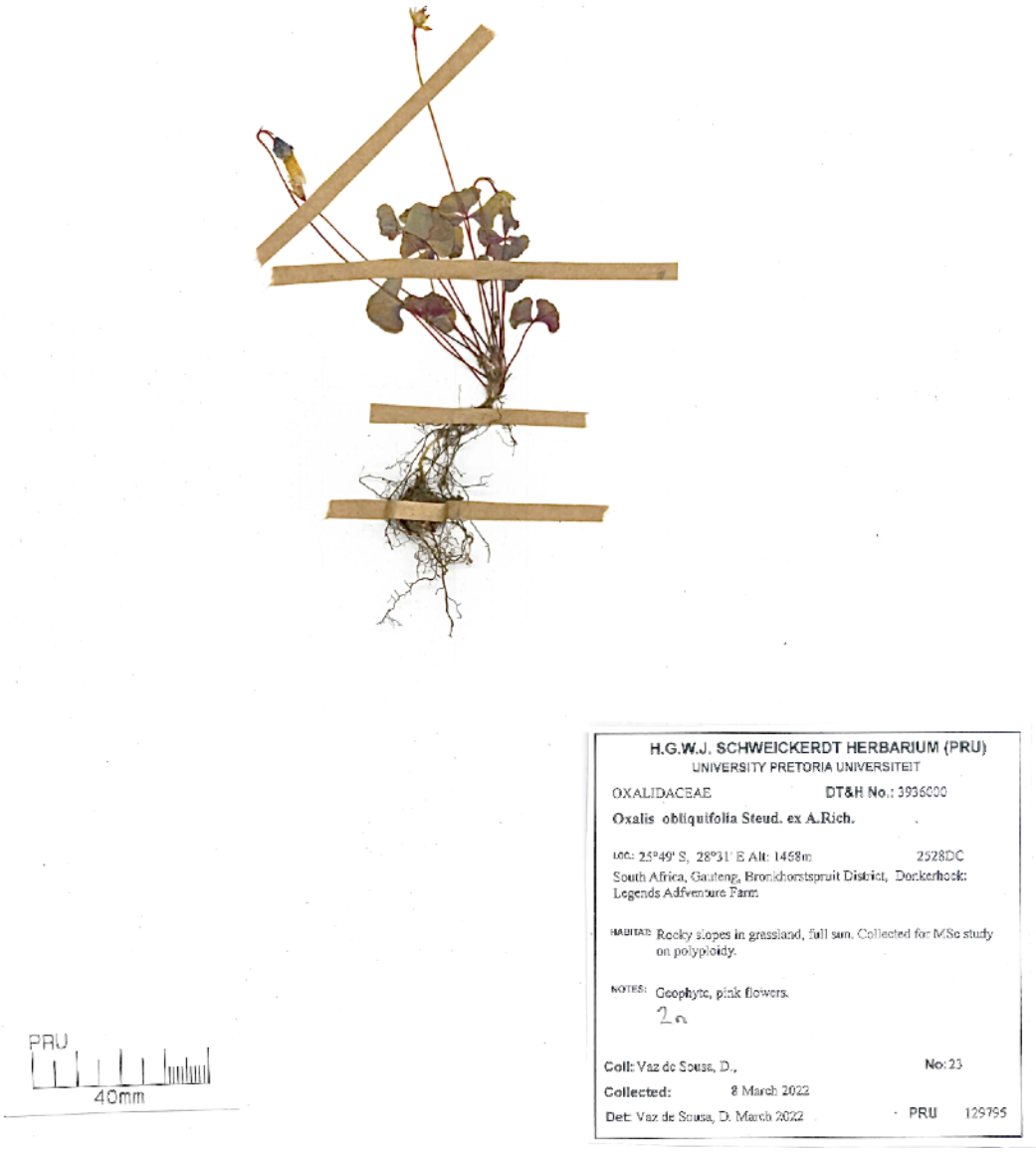
non-trivial seed set under artificial conditions. This raises many questions regarding the possible mechanisms through which reproductive isolation is maintained *in situ*, and how polyploids have become so successfully established within the existing set of diploid populations. This also highlights the inherent limitations to current taxonomic practices in recognising real diversity patterns in polyploid complexes, such as in *O. obliquifolia.*

The results of Chapter 2 suggest strong evidence of the Gigas effect in this system, with polyploids having larger leaves and flowers in common garden conditions. Larger flowers combined with some evidence of slight phenological shifts in flowering time, where polyploids tend to flower at the beginning of the season, provides compelling possibilities for pollinator mediated assortative mating as a potential prezygotic barrier to interploid crosses, although post-zygotic effects such as interploid seed fitness also need to be investigated. This may also suggest pollinator driven selection for particular reproductive characteristics that are favoured, thereby possibly reinforcing the prominent size differences observed in floral structure between diploids and polyploids. However, it remains to be seen whether the Gigas effect is still discernible in the wild, where environmental-induced size variation will also amplify variability in phenotypic expression, and thus also potentially obscure pollinator discrimination.

While there are some limits to the scope of this investigation, it is clear that African polyploid systems, such as explored here, have the potential to offer much insight regarding whole genome duplication and its ecological and evolutionary consequences. It provides a valuable system for the study of various aspects of polyploidy, as a major contributor to the evolution of angiosperms, and has the potential to contribute much to the existing literature on regional studies regarding the role of polyploidy in intraspecific diversity. In this context, the short-comings of this investigation can rather be viewed as exciting avenues for potential further investigations. In particular, pollinator interactions and intercytotype competition are two unexplored, yet potentially crucial biotic factors that may facilitate polyploid success in mixed-ploidy populations. The phenotypic differences observed in this investigation, and the potential associated physiological consequences of these, may have profound effects on polyploid competitive ability, and pollinator interactions. Both of these biotic factors, ecological niche shifts and direct competition, provide promising directions of enquiry for further investigations into the intricate mechanisms underlying this complex system.

APPENDICES

Appendix 1A: Voucher specimen of a diploid individual, *Oxalis obliquifolia,* found near Donkerhoek (east Gauteng). PRU129795.



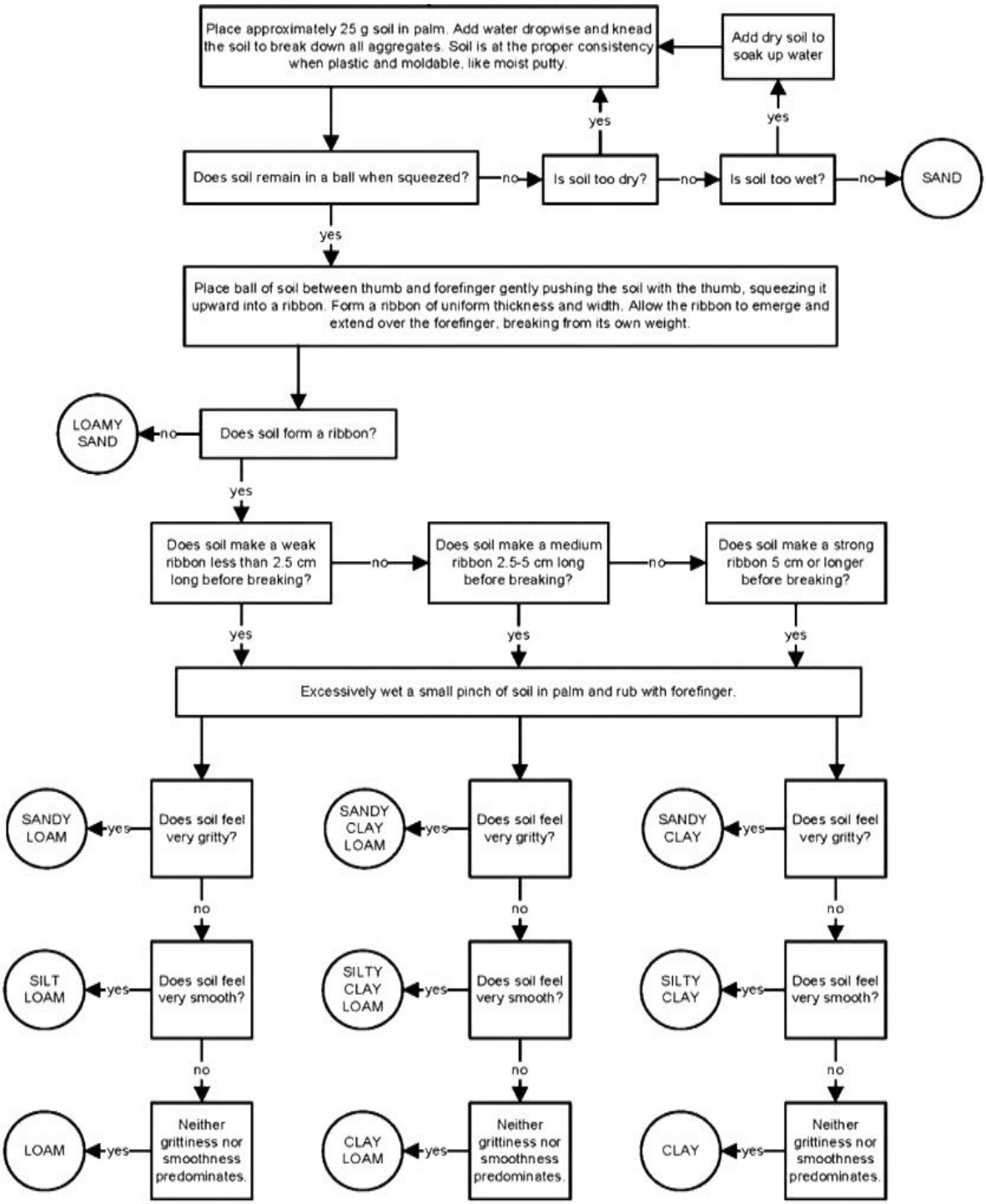
Appendix 1B: List of *Oxalis obliquifolia* voucher specimens, cytotype and associated call numbers and locality details for each site.

Sample number	Site	Local Municipality	Locality description	Cytotype	Geographic coordinates	PRU* call number
OA015	Sable Ranch	Mogale City	Along hiking trail on rocky slope	Diploid (2n)	S25.93487 E27.61824	PRU129335
OB001	Miertjie le Roux	City of Tshwane	Along side of road on the farm	Tetraploid (4n)	S25.78102 E28.54816	PRU128128
OC015	Carlswald Estate	City of Johannesburg	Next to jogging path in grassland	Tetraploid (4n)	S25.97627 E28.10171	PRU129949
OD001	Klipkraal Trails	Midvaal	Along boundary fence on farm	Tetraploid (4n)	S26.79635 E28.22789	PRU129942
OE001	Krugersdorp	Mogale City	Along hiking trail in open grassland	Tetraploid (4n)	S26.04579 E27.78981	PRU129943
OF001	Faerie Glen	City of Tshwane	Along hiking trail in open grassland	Diploid (2n)	S25.7742 E28.29369	PRU128127
OG001	Magaliesburg	City of Tshwane	Along hiking trail on rocky slope	Diploid (2n)	S25.80123 E27.99029	PRU129796
OH003	Hazeldean Trails	City of Tshwane	Along hiking trail in open grassland	Tetraploid (4n)	S25.77355 E28.40455	PRU129951
OI008	Alberton	City of Johannesburg	On rocky slope in open grassland	Tetraploid (4n)	S26.30157 E28.07494	PRU129944
OJ001	Fochville	Merafong City	Found on base of rocky outcrop.	Diploid (2n)	S26.56123 E27.50775	PRU129945
OK001	Kloofendal Nature Reserve	City of Johannesburg	Along hiking trail on rocky slope	Tetraploid (4n)	S26.13077 E27.88219	PRU129952
OL008	Olifantsfontein	City of Johannesburg	Found in open grassland	Diploid (2n)	S25.94517 E28.17904	PRU129946
OM001	Moreleta Kloof Nature Reserve	City of Tshwane	Along hiking trail in open grassland	Diploid (2n)	S25.81608 E28.28964	PRU129950
ON001	Klipreviersberg Nature Reserve	City of Johannesburg	Along hiking trail on rocky slope	Hexaploid (6n)	S26.303649 E28.012772	PRU129953
OO001	Cradle Moon Lakeside Lodge	Mogale City	Found in open grassland	Tetraploid (4n)	S25.95757 E27.86028	PRU129947
OP001	University of Pretoria Grassland	City of Tshwane	Found in open grassland	Tetraploid (4n)	S25.74191 E28.26113	*

Sample number	Site	Local Municipality	Locality description	Cytotype	Geographic coordinates	PRU* call number
OQ009	Windy Brow Game Reserve	City of Tshwane	On hiking trail in grassland	Tetraploid (4n)	S25.68804 E28.50303	PRU129794
OR004	Sandton	City of Johannesburg	Found on rocky slope	Tetraploid (4n)	S26.03108 E28.04206	PRU129954
OS004	Smuts Koppie	City of Tshwane	Along hiking trail on rocky slope	Tetraploid (4n)	S25.8913 E28.23862	PRU128130
OT001	Muningi Gorge	City of Tshwane	Found in open grassland	Diploid (2n)	S25.57704 E28.59107	PRU129334
OU001	Legends Adventure Farm	City of Tshwane	Next to dirt-road	Diploid (2n)	S25.82375 E28.55128	PRU129795
OV001	Kempton Park	City of Ekurhuleni	Next to bike trail in open grassland	Tetraploid (4n)	S26.06965 E28.26629	PRU129797
OW001	Wonderboom east	City of Tshwane	Along hiking trail on rocky slope	Tetraploid (4n)	S25.69308 E28.20577	PRU128129
OX008	Happy Acres	Mogale City	Along hiking trail on rocky slope	Tetraploid (4n)	S26.02610 E27.54644	PRU130792
OY001	Suikerbosrand Nature Reserve	Midvaal	On rocky slope in open grassland	Hexaploid (6n)	S26.48166 E28.21008	PRU129948

* Missing PRU numbers will be added when reproductive material becomes available for submission

Appendix 1C: Copy of the flow diagram used in the soil texture characterisation of soil samples collected for each individual *Oxalis obliquifolia* individual collected across Gauteng, created by Thien (1979).



Appendix 1D: R script for Multiple Factor Analysis (MFA) and PerMANOVA of abiotic variables associated with cytotype distribution patterns.

Install packages
> install.packages("FactoMineR")
> install.packages("Factoshiny")
> install.packages("cluster")
> install.packages("vegan")

Add libraries
> library(FactoMineR)
> library(Factoshiny)
> library(readxl)
> library(cluster)
> library(vegan)

Load and view data from excel table
> read_excel("FileName.xlsx")
> ObjectName <- read_excel("FileName.xlsx")
> View(ObjectName)

MFA Analysis

Prepare data for analysis, identify columns/variables and assign to object
> DF <- ObjectName[,c("Cytotype", "Ploidy", "Site", "Elevation", "Northness", "Slope", "MinTemp", "MaxTemp", "Precipitation", "SoilGravel", "Geology", "SoilTexture", "SunShade")]

Identify groups of variables, assigned variable types (categorical ("n") or continuous ("s"), run MFA and assign out-put to object
> res.MFA <- MFA(DF, group = c(2, 1, 3, 3, 1, 2, 1), type = c("n", "n", "s", "s", "s", "n", "n"), name.group = c("Cytotype" ,"Site" ,"Topography" ,"Climate" ,"Substrate" ,"Soil" ,"Exposure"), num.group.sup = c(1, 2), graph = FALSE)

Plot MFA, individuals labelled by cytotype
> plot.MFA(res.MFA, choix = "ind", lab.par = FALSE, invisible = c('quali', 'quali.sup'), habillage = 'Cytotype', title = "Individual factor map")

Plot variables
> plot.MFA(res.MFA, choix = "var", habillage = 'group', title = "Correlation circle")

Plot partial axes plot
> plot.MFA(res.MFA, choix = "axes", habillage = 'group')

- -

PerMANOVA Analysis

Isolate abiotic variables
> DF2 <- DF[,3:13]

Check for autocorrelation using Pearson's correlation matrix
> round(cor(Df2, method = "pearson"), digits = 2)

Remove autocorrelated variables
> DF2[, -4: -5]

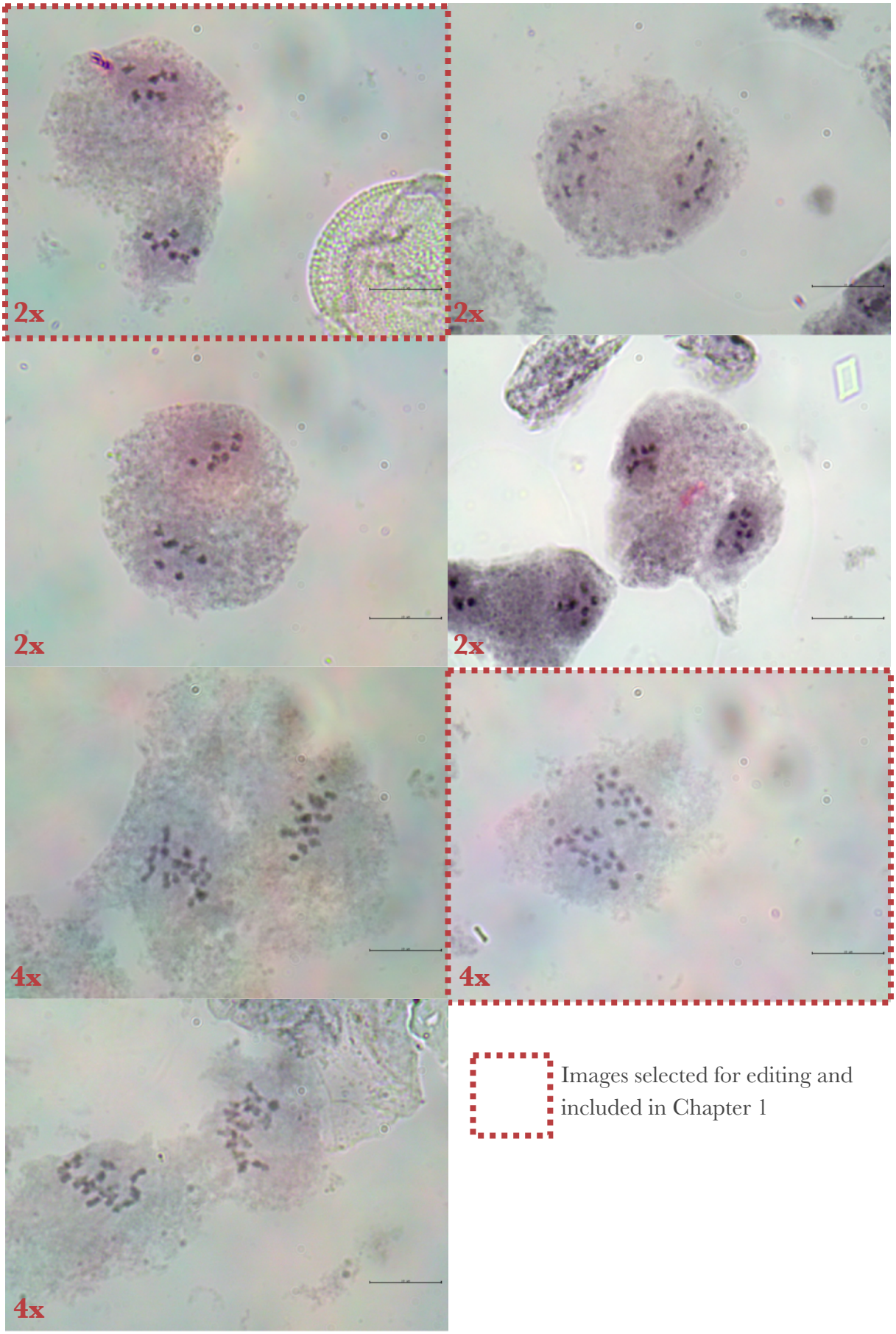
Create Gower's distance matrix
> Dist1 <- daisy(DF2, metric = c("gower"))

Run PerMANOVA
> adonis2(Dist1 ~ Cytotype*Site, data = DF)

Appendix 1E: Data matrix of abiotic variables (climate, topology and substrate) for each individual plants mapped and cytotyped

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_1E_AbioticVariables.xlxs"

Appendix 1F: Original size and colour microscope images of chromosome squashes used to determine chromosome number and morphology in *Oxalis obliquifolia*.



Appendix 1G: Abiotic variable contribution to construction of MFA dimensions 1 and 2

Table 1G: The contribution of active groups of abiotic variables to the construction of dimensions 1 and 2 of the MFA, and the explanatory power/ association of supplementary variables to those dimensions

Variable Groups	Contribution to Dim 1	Contribution to Dim 2
Active		
Topography - Elevation - Northness - Slope	30.968	15.552
Climate - Minimum temperature - Maximum temperature - Mean annual precipitation	**37.953**	27.099
Substrate - Underlying Geology - Soil texture	1.521	4.395
Soil -Percentage of coarse fragments	27.155	**40.034**
Exposure - Sun vs Shade	2.402	12.920
Supplementary		
Cytotype	0.156	0.029
Site	**0.939**	**0.837**

Appendix 2A: Morphological data of foliar and floral characters captured for all individuals included in the common garden experiment, captured at the peak of the growing season.

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_2A_MorphologyData_Peak.xlxs"

Appendix 2B: Morphological data of foliar and floral characters captured for each individual included in the common garden experiment, captured 2 months after emergence.

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_2B_MorphologyData_2Months.xlxs"

Appendix 2C: Colour charts used for the categorisation of floral and abaxial leaf surface colours

Appendix 2D: Vegetative phenology data for each individual included in the common garden experiment.

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_2D_VegetativePhenology.xlxs"

Appendix 2E: Flower phenology data for 31 individuals (including diploids, tetraploids and hexaploids) included in the common garden experiment.

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_2E_FlowerPhenology.xlxs"

Appendix 2F: R script for univariate analyses of morphological traits associated with different cytotypes.

Install package
> install.packages("multcompView")
>install.packages("MASS")
> install.packages("multcomp")

Add libraries
> library(multcompView)
> library(readxl)
> library(MASS)
> library(multcomp)

Load and view data from excel table
> read_excel("FileName.xlsx")
> ObjectName <- read_excel("FileName.xlsx")
> View(ObjectName)

Check for autocorrelation using Pearson's correlation matrix
> round(cor(ObjectName[,c(5:27)], method = "pearson"), digits = 2)

- -

GLM Analysis

Perform GLM
> GLM1 <- glm(PredictorVariable ~ Cytotype, family = c("Gamma", "poisson", "quasipoisson", "gaussian", "negbin"), data = ObjectName)
> summary(GLM1)

Extract p-values

```
> P1 <- summary(GLM1)$coef[, "Pr(>|t|)"]

#### Adjust p-values ####
> p.adjust(P1, method = "BH")

#### Turkey post-hoc test ####
>comps1 <- glht(GLM1, linfct = mcp(Cytotype = "Tukey"))
> summary(comps1)
>cld(comps1)
```

Appendix 2G: Selection of distribution family for each GLM performed with morphological traits as predictors of cytotype

Table 2G: Distribution family selection for GLM analyses of morphological traits, indicating individual traits, approximate lambda values and result of Shapiro-Wilk test, data transformations, possible distribution families, AIC values and residual deviance values, for each parameter combination.

Trait	**Transformation** None Log/BoxCox	**GLM family**	**AIC**	**Residual deviance** on 95 df
Foliar traits				
Middle leaflet length (mm)	None	Gaussian	477.25	689.11
		InverseGaussian	462.89	0.19385
		Gamma	464.79	2.8587
Continuous data	**Log**	Gaussian	-63.757	2.7590
Lambda = 0		InverseGaussian	-64.02	**0.14476**
ShapiroWilk=0.0003349		**Gamma**	**-64.365**	0.38472
Middle leaflet width (mm)	None	Gaussian	524.29	1113.6
		InverseGaussian	531.95	0.14242
Continuous data		Gamma	525.49	2.7036
Lambda = 0.6	**Log**	**Gaussian**	**-64.129**	2.7486
ShapiroWilk= 0.5975		InverseGaussian	-55.84	**0.11015**
		Gamma	-59.149	0.31992
	Sqrt	Gaussian	91.701	13.480
		InverseGaussian	97.727	0.15589
		Gamma	94.708	0.68149
Lateral leaflet length (mm)	None	Gaussian	435.46	449.88
		InverseGaussian	440.98	0.19849
		Gamma	436.55	2.5282
Continuous data	**Log**	**Gaussian**	**-73.479**	2.4985
Lambda = 0.45		InverseGaussian	-66.917	**0.15458**
ShapiroWilk=0.0525		Gamma	-69.593	0.38862
	Sqrt	Gaussian	43.519	8.2444
		InverseGaussian	47.64	0.17560
		Gamma	45.594	0.62847

Trait	Transformation None Log/BoxCox	GLM family	AIC	Residual deviance on 95 df
Lateral leaflet width (mm)	None	Gaussian	477.71	692.35
		InverseGaussian	479.12	0.14840
Continuous data		Gamma	475.68	2.3959
Lambda = 0.45	**Log**	**Gaussian**	**-76.137**	2.4316
ShapiroWilk=0.2225		InverseGaussian	-70.809	**0.11536**
		Gamma	-73.095	0.31684
	Sqrt	Gaussian	63.019	10.059
		InverseGaussian	65.351	0.14974
		Gamma	63.801	0.60332
Petiole length (mm)	None	Gaussian	895.43	49145
		InverseGaussian	913.06	0.083110
Continuous data		Gamma	902.51	6.5772
Lambda = 1	**Log**	**Gaussian**	**28.544**	7.0761
ShapiroWilk=0.6452		InverseGaussian	37.295	**0.086003**
		Gamma	34.053	0.37259
Number of leaves	**None**	Poisson	594.1	160.85
		Neg binomial	**574.63**	**89.835**
Count Data				
Ratio middle leaflet width to length	Log	quasiPoisson (Negative values present)	-	-
MidLeafWidth/ MidLeafLength	**None**	**quasiPoisson**	**NA**	**1.8221**
Ratio lateral leaflet width to length	Log	quasiPoisson	NA	1.8384
	None	**quasiPoisson**	NA	**1.0081**
LatLeafWidth/LatLeafLength				
Floral traits				
Petal width (mm)	None	Gaussian	369.61	229.74
		InverseGaussian	367.7	0.25095
Continuous data		Gamma	365.82	2.3655
	Log	**Gaussian**	**-77.446**	2.3993
Lambda = +- 0.35		InverseGaussian	-72.552	0.21920
ShapiroWilk= 0.01106		Gamma	-74.818	0.48316
	Sqrt	Gaussian	8.4678	5.7653
		InverseGaussian	9.0781	**0.19361**
		Gamma	8.2309	0.59558
Petal length (mm)	None	Gaussian	451.76	531.28
		InverseGaussian	453.53	0.098275
Continuous data		Gamma	451.34	1.6921
	Log	**Gaussian**	**-109.26**	1.7342
Lambda = 0.65		InverseGaussian	-106.26	**0.07595**
ShapiroWilk= 0.1192		Gamma	-107.47	0.21492
	Sqrt	Gaussian	34.214	7.4976
		InverseGaussian	36.103	0.10303
		Gamma	35.073	0.42807

Trait	Transformation None Log/BoxCox	GLM family	AIC	Residual deviance on 95 df
Flower diameter (mm)	None	Gaussian	516.97	1033.5
		InverseGaussian	514.35	0.12054
Continuous data		Gamma	513.03	2.4028
	Log	**Gaussian**	**-76.819**	2.4147
Lambda = 0.15		InverseGaussian	-74.332	**0.091377**
ShapiroWilk= 0.03588		Gamma	-75.424	0.27132
Sepal length (mm)	None	Gaussian	279.93	92.013
		InverseGaussian	278.82	**0.39404**
Continuous data		Gamma	277.87	2.3843
	Log	**Gaussian**	**-77.737**	2.3922
Lambda =+- 0.15		InverseGaussian	-73.825	0.42292
ShapiroWilk=9.803e-06		Gamma	-75.603	0.74896
Sepal width (mm)	None	Gaussian	148.75	24.128
		InverseGaussian	147.37	**1.5458**
Continuous data		Gamma	147.53	3.8261
	Log	Gaussian	-30.744	3.8641
Lambda = +- 0.15		InverseGaussian	-31.164	5.5110
ShapiroWilk= 1.91e-13		**Gamma**	**-31.271**	4.8436
Bract length (mm)	None	Gaussian	386.69	273.49
		InverseGaussian	369.13	**1.9302**
Continuous data		Gamma	371.92	9.5950
	Log	**Gaussian**	**56.3**	9.3928
Lambda = -0.1		InverseGaussian	61.535	2.6137
ShapiroWilk= 3.759e-06		Gamma	57.792	3.8968
Peduncle length (mm)	None	Gaussian	882.22	42946
		InverseGaussian	881.85	**0.044258**
Continuous data		Gamma	879.93	4.2603
	Log	**Gaussian**	**-20.118**	4.3067
Lambda =+- 0.35		InverseGaussian	-18.135	0.045492
ShapiroWilk=0.1115		Gamma	-18.905	0.20703
	Sqrt	Gaussian	293.38	105.54
		InverseGaussian	294.94	0.10907
		Gamma	293.9	1.0711
Ratio flower diameter to petal length	Log	quasiPoisson (Negative nalues)	-	-
	None	**quasiPoisson**	**NA**	**1.5685**
FlowerDiameter/PetalLength				
Ratio petal length to width	Log	quasiPoisson	NA	3.1530
	None	**quasiPoisson**	**NA**	**2.6246**
PetalLength/PetalWidth				
Ratio sepal length to width	**Log**	**quasiPoisson**	**NA**	**4.2967**
	None	quasiPoisson	NA	9.3694
SepalLength/SepalWidth				

Trait	Transformation None Log/BoxCox	GLM family	AIC	Residual deviance on 95 df
Ratio peduncle length to bract position PeduncleLength/ BractPosition	Log **None**	quasiPoisson **quasiPoisson**	NA **NA**	3.0233 **0.56499**
Number of inflorescences Count data	**None**	Poisson **Neg binomial**	519.31 **504.99**	161.90 **97.815**
Difference in peduncle and petiole length (mm) Continuous data, with negative values Lambda= Must be positive ShapiroWilk=0.7235	**None** Log (Na's produced)	**Gaussian** InverseGaussian (negative values) Gamma (negative values) Gaussian InverseGaussian Gamma	**893.87** - - - - -	**48368** - - - - -

Appendix 2H: R script for multivariate analyses of morphological traits associated with different cytotypes.

Install packages
> install.packages("FactoMineR")
> install.packages("Factoshiny")
> install.packages("MASS")
> install.packages("ggplot2")
> install.packages("ggfortify")
> install.packages("rlang")
> install.packages("caret")

Add libraries
> library(FactoMineR)
> library(Factoshiny)
> library(readxl)
> library(MASS)
> library(ggplot2)
> library(ggfortify)
> library(rlang)
> library(caret)

Load and view data from excel table
> read_excel("FileName.xlsx")
> ObjectName <- read_excel("FileName.xlsx")

```
> View(ObjectName)

-----------------------------------------------------------------------
                    ##### Factor Analysis of Mixed Data #####

#### Prepare data for analysis, identify columns/variables and assign to object ####
> DF <- ObjectName[,c("Accession", "Cytotype", "FlowerColour", "LeafAbaxialColour",
"PetioleLength", "MiddleLeafletLength", "MiddleLeafletWidth", "LateralLeafletLength",
"LateralLeafletWidth", "FlowerDiameter", "PetalLength", "PetalWidth", "SepalLength",
"SepalWidth", "NumberOfLeaves", "NumberOfInfloresecneces", "BractLength", "PeduncleLength",
"BractPosition", "RatioBractPositionToPeduncleLength", "RatioSepalLengthToSepalWidth",
"RatioPetalLengthToPetalWidth", "RatioSepalLengthToPetalLength",
"RatioLateralLeafletWidthToLength", "RatioMidLeafletWidthToLength",
"DifferencePeduncleAndPetioleLength", "RatioFlowerDiameterToPetalLength" )]

#### Conduct FAMD, identify supplementary variables ####
res.FAMD<-FAMD(DF, sup.var=c(1,2),graph=FALSE)


#### Plot MFA, individuals labelled by cytotype #####
>  plot.FAMD(res.FAMD,invisible=c('quali','quali.sup','ind.sup'),habillage=2,title="Graph  of
individuals and categories")

#### Plot variables ####
> plot.FAMD(res.FAMD,axes=c(1,2),choix='var',title="Graph of the variables")

#### Plot Correlation circle ####
> plot.FAMD(res.FAMD, choix='quanti',title="Correlation circle")


-----------------------------------------------------------------------
                    ##### Principle Component Analysis #####

#### Conduct PCA, identify columns/variables and assign to object ####
> pca_res <- prcomp(ObjectName[5:27], scale. = TRUE)

#### View Output ####
> pca_res

#### Plot PCA with ellipses ####
PCA1 <- autoplot(pca_res, data = ObjectName, colour = 'Cytotype', size=1.0) + theme_light() +
stat_ellipse(geom = "polygon", aes(x=PC1, y=PC2, color= Cytotype, fill= Cytotype), type = "norm",
level = 0.95, alpha = 0.25)
```

- -

```
##### Linear Discriminant Analysis #####

#### Prepare data for analysis, identify columns/variables and assign to object ####
> ObjectName <- read_excel("FileName.xlsx", col_types = c("skip", "text", "skip", "skip", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric", "numeric"))

#### LDA Step 1, set random seed ####
> set.seed(123)
#### Create data partition and training and test data ####
> training.samples <- createDataPartition(ObjectName$Cytotype, p = 0.8, list = FALSE)
> train.data <- ObjectName[training.samples, ]
> test.data <- Object[-training.samples, ]

#### Data preprocessing and tranformation ####
> preproc.param <- preProcess(train.data, method = c("center", "scale"))
> train.transformed <- predict(preproc.param, train.data)
> test.transformed <-  predict(preproc.param, test.data)

#### Fit the LDA model, view model output ####
> model <- lda(Cytotype~., data = train.transformed)
> model

#### Use model to make predictions ####
> predictions <-  predict(model, test.transformed)

#### Find model accuracy ####
> mean(predictions$class==test.transformed$Cytotype)

#### Plot LDA ####
> plot(model)
> lda_plot <- cbind(train.data, predict(model)$x)
> PlotLDA <- ggplot(lda_plot, aes(LD1, LD2)) + geom_point(aes(color = Cytotype), size = 1)
```

Appendix 2I: Factor Analysis of Mixed Data (FAMD) based on 23 quantitative and 2 qualitative morphological characters as predictors of cytotype

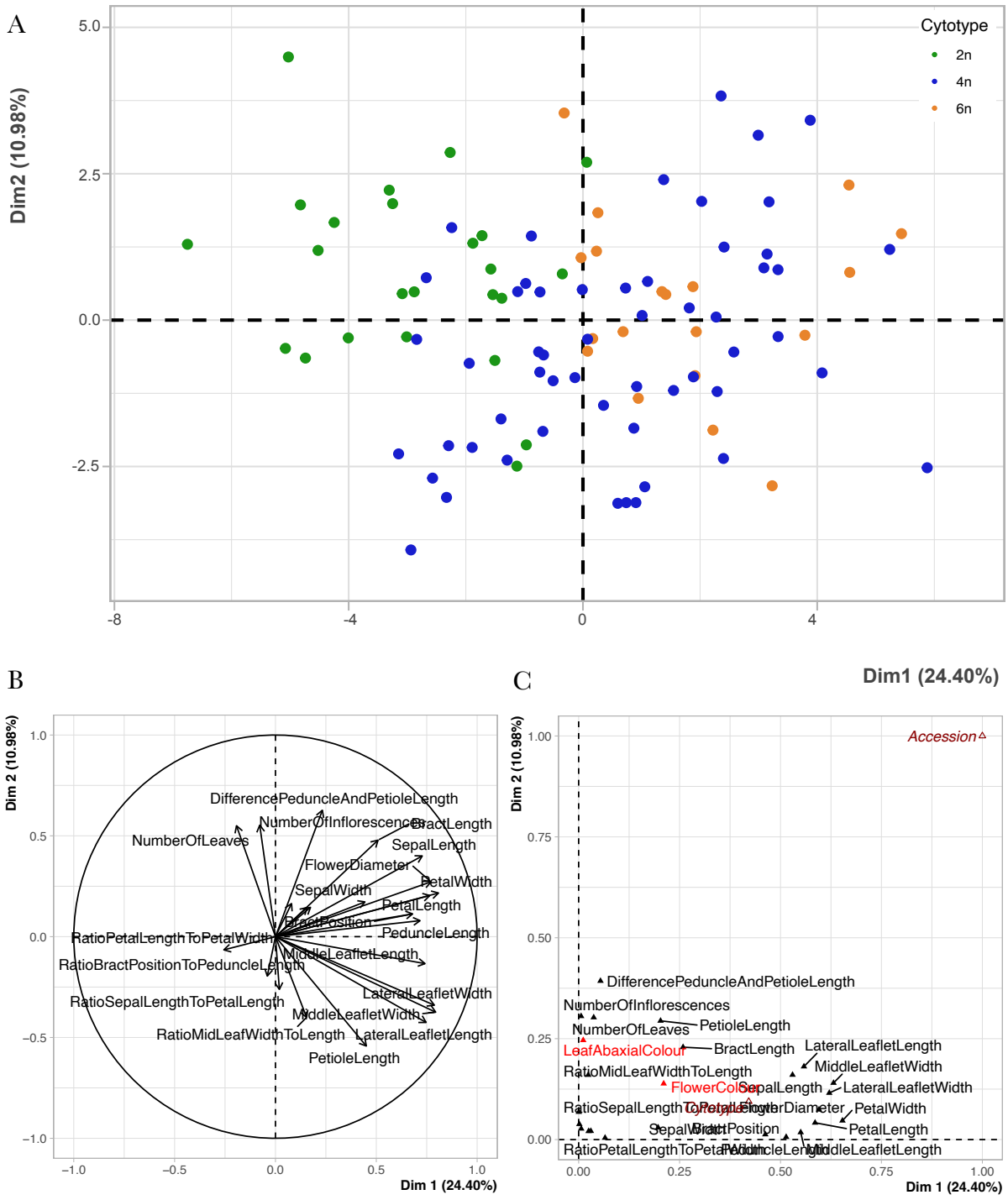


Figure 2I: A- Factor Analysis of Mixed Data (FAMD) based on 23 quantitative and 2 qualitative morphological characters as predictors of cytotype of *Oxalis obliquifolia*, with dimensions 1 and 2 accounting for a cumulative 35.38 % of the variation observed. **B-** Correlation circle showing the 23 continuous variables used in the construction of the FAMD. **C-** Graph of all variables used in the construction of dimensions 1 and 2 of the FAMD (quantitative variables in black; qualitative variables in red; and supplementary variables in brown).

Appendix 2J: R script for univariate analyses of plant phenology associated with different cytotypes.

```
#### Perform GLM with poisson distribution ####
> GLM1 <- glm(PredictorVariable ~ Cytotype, family = c("poisson"), data = ObjectName)
> summary(GLM1)

#### Extract p-values ####
> P1 <- summary(GLM1)$coef[, "Pr(>|z|)"]

#### Adjust p-values ####
> p.adjust(P1, method = "BH")

#### Turkey post-hoc test ####
>comps1 <- glht(GLM1, linfct = mcp(Cytotype = "Tukey"))
> summary(comps1)
>cld(comps1)
```

Appendix 2K: Comparison of the results of the PCA conducted on the two data sets obtained for the morphological characters measured in the common garden experiment, at both the peak of the growing season and 2 months after each individual emerged. Similarities in the identification of important variable in the construction of PC1, PC2 and PC3 are marked in bold, and difference have been highlighted in bold and red.

Table 2K.1: Loading scores for variables contributing to the first 3 principle components of the PCA constructed from morphological data for all 98 individuals, captured 2 months after emergence of each individual.

	PC1	PC2	PC3
Petiole length	0.171148589	**-0.32927082**	-0.138966729
Middle-leaflet length	0.290841467	-0.15621006	-0.009598323
Middle-leaflet width	**0.310520246**	-0.19435230	-0.176017614
Lateral-leaflet length	0.290749181	-0.28680919	-0.101817525
Lateral-leaflet width	**0.308723684**	-0.17774711	-0.178682023
Flower diameter	0.297529101	0.14141799	0.110744776
Petal length	0.296485312	-0.01811135	0.258939692
Petal width	**0.314362768**	0.18323322	-0.001857691
Sepal length	0.286328271	0.14537488	0.172273254
Sepal width	0.173880675	0.26341731	-0.256890841
Number of leaves	-0.064900053	0.19270049	0.186667870

Number of inflorescences	-0.023660230	0.22021498	0.167104357
Bract length	0.202633896	0.13133874	0.261332948
Peduncle length	0.276671723	0.07974962	0.017381272
Bract position	0.261832162	0.04565799	0.111569304
Ratio bract position to peduncle length	-0.012749293	0.08574978	**-0.375834705**
Ratio sepal length to sepal width	0.068636915	-0.15450242	**0.405244030**
Ratio petal length to petal width	-0.100290763	**-0.30953206**	**0.335876393**
Ratio sepal length to petal length	0.001668905	-0.20652811	0.100764667
Ratio lateral-leaflet width to length	0.035361868	0.22224862	-0.172582874
Ratio mid-leaflet width to length	0.058144160	-0.06960918	-0.263220593
Difference peduncle and petiole length	0.092449205	**0.41093360**	0.157773477
Ratio flower diameter to petal length	0.057736119	0.25886646	-0.190038000

Table 2K.2: Loading scores for variables contributing to the first 3 principle components of the PCA constructed from morphological data for all 98 individuals, captured at the peak of the growing season.

	PC1	PC2	PC3
Petiole length	-0.127870320	-0.04480974	0.33817611
Middle-leaflet length	-0.284249269	-0.23640178	0.19503517
Middle-leaflet width	**-0.315388724**	-0.17399790	0.05877109
Lateral-leaflet length	-0.304269668	-0.13443735	0.18261790
Lateral-leaflet width	-0.304414724	-0.18844770	0.06862474
Flower diameter	**-0.322699755**	0.10536028	-0.08090536
Petal length	-0.306061652	0.20348656	0.04212910
Petal width	**-0.314438235**	0.04123640	-0.10125485
Sepal length	-0.223831398	-0.13372437	0.09380340
Sepal width	-0.171509972	-0.07105417	**-0.39801765**
Number of leaves	0.125022750	**-0.37049037**	-0.03180551
Number of inflorescences	0.037045335	**-0.39484893**	-0.07322786
Bract length	-0.214147942	-0.14106976	0.12145883
Peduncle length	-0.263035552	0.17899111	-0.07203242

Bract position	-0.247176052	0.19678950	-0.05229351
Ratio bract position to peduncle length	0.027557085	0.11802490	0.09320373
Ratio sepal length to sepal width	-0.003693819	-0.02917894	**0.43118647**
Ratio petal length to petal width	0.116146820	0.26172076	0.26685831
Ratio sepal length to petal length	0.107686627	**-0.40244182**	-0.02568226
Ratio lateral-leaflet width to length	-0.013229607	-0.10776932	-0.24583070
Ratio mid-leaflet width to length	-0.099747786	0.09063359	-0.23251528
Difference peduncle and petiole length	-0.111256226	0.19461109	**-0.36708409**
Ratio flower diameter to petal length	-0.020699924	-0.30713541	-0.27480430

Continued on next page.

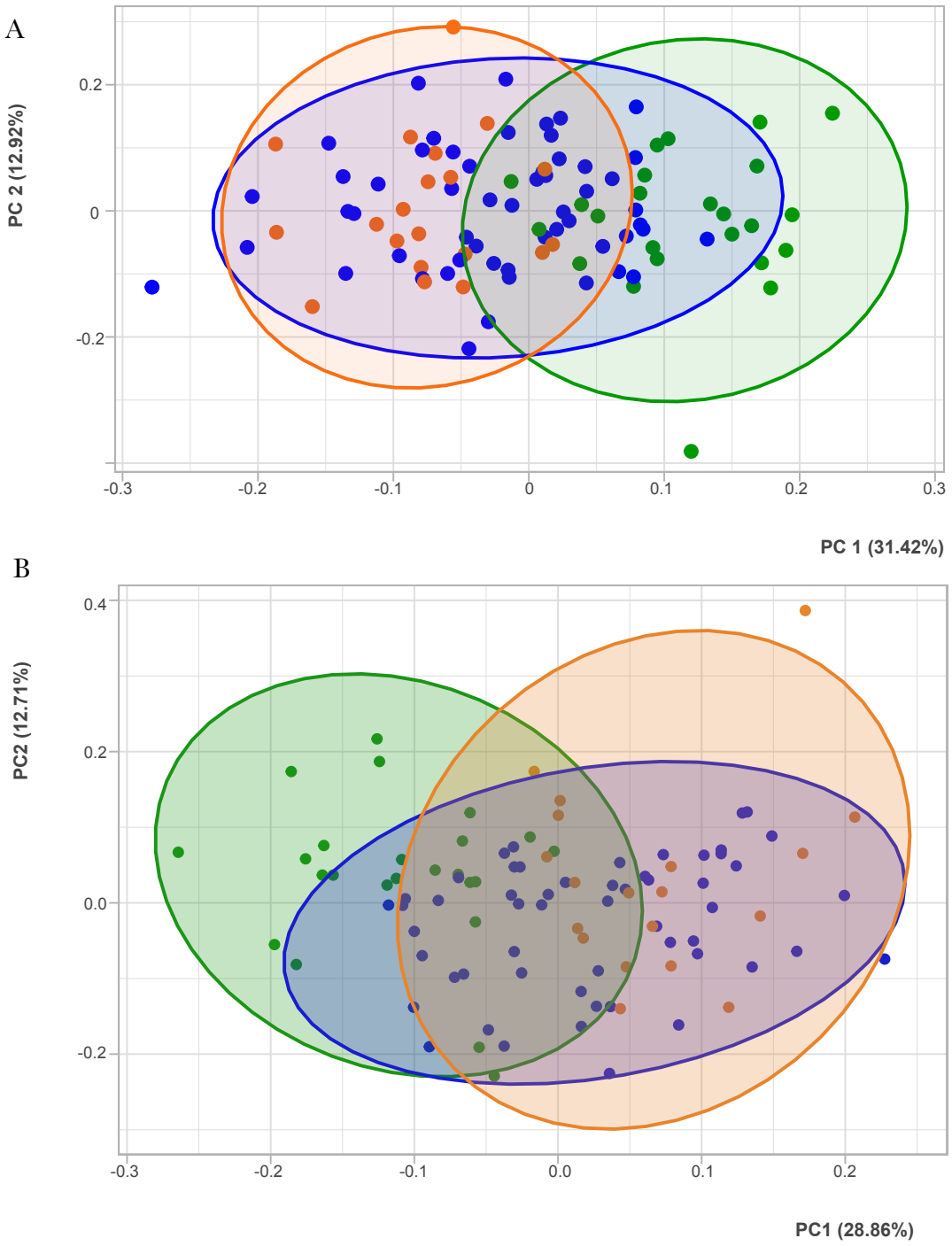


Figure 2K: A- Principle component analysis (PCA) based data set of character terms measured at the peak of the growing season. **B-** PCA based on data set for characters measured after 2 months from emergence of each individual plant. PCAs based on 23 morphological traits as predictors of cytotype (diploid - green, tetraploid - blue, hexaploid - orange).

Appendix 3A: Crossing data

DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**
File name: "Appendix_3A_CrossData.xlxs"

Appendix 3B: R script for hurdle model analysis of success rate and seed set associated with the interaction of different types of crosses (self-pollinated, within cytotype, between cytotype) and maternal cytotypes (diploids, tetraploids, hexaploids).

```
#### Install packages ####
> install.packages("pscl")
> install.packages("emmeans")

#### Add libraries ####
> library(pscl)
> library(emmeans)

#### Load and view data from excel table ####
> read_excel("FileName.xlsx")
> ObjectName <- read_excel("FileName.xlsx")
> View(ObjectName)

----------------------------------------------------------------------
                    ##### Hurdle Analysis #####

#### Fit the hurdle model with negative binomial distribution, view model output ####
>hurdle1 <- hurdle(NumberOfSeeds ~ TypeOfCross*MaternalCytotype, data = ObjectName, dist =
"negbin", zero.dist =  c("binomial"))
> summary(hurdle1)

#### Turkey post-hoc tests, back transformations and confidence intervals ####
> emmeans(hurdle1,~TypeOfCross*MaternalCytotype, mode = c("zero")))
> cld(emmeans(hurdle1,~TypeOfCross*MaternalCytotype, mode = c("zero")))

> emmeans(hurdle1,~TypeOfCross*MaternalCytotype, mode = c("count")))
> cld(emmeans(hurdle1,~TypeOfCross*MaternalCytotype, mode = c("count")))
```

Appendix 3C: Genbank alignment- ITS sequence data- Accession numbers

Link to be included once sequences released to public on GenBank

Sample ID.	Accession Number
OF016_2n	OP782704
OF076_2n	OP782705
OF086_2n	OP782706
OF091_2n	OP782707
OF096_2n	OP782708
OF099_2n	OP782709
OF100_2n	OP782710
OG001_2n	OP782711
OG003_2n	OP782712
OG008_2n	OP782713
OJ002_2n	OP782714
OJ004_2n	OP782715
OJ006_2n	OP782716
OL008_2n	OP782717
OL009_2n	OP782718
OL010_2n	OP782719
OL011_2n	OP782720
OL013_2n	OP782721
OL014_2n	OP782722
OL015_2n	OP782723
OL017_2n	OP782724
OM018_2n	OP782725
OM020_2n	OP782726
OM021_2n	OP782727
OM024_2n	OP782728
OS003_2n	OP782729
OS010_2n	OP782730
OS015_2n	OP782731
OS00Q_2n	OP782732
OX005_2n	OP782733
OX006_2n	OP782734
OX013_2n	OP782735
OB003_4n	OP782736
OB007_4n	OP782737
OB014_4n	OP782738
OB018_4n	OP782739
OB020_4n	OP782740
OD001_4n	OP782741
OD009_4n	OP782742
OD012_4n	OP782743
OD017_4n	OP782744

OF056_4n	OP782745
OF071_4n	OP782746
OF101_4n	OP782747
OF102_4n	OP782748
OF103_4n	OP782749
OH001_4n	OP782750
OH002_4n	OP782751
OH004_4n	OP782752
OH005_4n	OP782753
OI008_4n	OP782754
OI011_4n	OP782755
OI014_4n	OP782756
OI015_4n	OP782757
OJ014_4n	OP782758
OK005_4n	OP782759
OK013_4n	OP782760
OK027_4n	OP782761
OK032_4n	OP782762
OL001_4n	OP782763
OL004_4n	OP782764
OS001_4n	OP782765
OS010Q_4n	OP782766
OW005b_4n	OP782767
OW008_4n	OP782768
OW00Qb_4n	OP782769
OX007_4n	OP782770
OX010_4n	OP782771
OB006_6n	OP782773
OD003_6n	OP782774
OD004_6n	OP782775
OD005_6n	OP782776
OD006_6n	OP782777
OI002_6n	OP782778
OI007_6n	OP782779
OL002_6n	OP782780
OL003_6n	OP782781
OL007_6n	OP782782
ON014_6n	OP782783
ON021_6n	OP782784
ON025_6n	OP782785

Appendix 3D: Arlequin input codes

Appendix 3D-1 Grouped by Site : DOI: 10.25403/UPresearchdata.21509226
Appendix 3D-2 Grouped by Cytotype : DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**

Appendix 3E: Summary of data spread and sampling for crosses performed

Number of crosses with maternal cytotypes:

	2x	4x	6x
Self-pollinated	102	112	77
Within-cytotype	93	109	81
Between-cytotype	238	277	51

Number of crosses with paternal cytotypes:

	2x	4x	5x	5x+	6x
Self-pollinated	102	112	0	0	77
Within-cytotype	93	109	0	0	81
Between-cytotype	108	109	70	75	204

Legitimate cross-cytotype combinations:

	Maternal 2x	Maternal 4x	Maternal 6x
Paternal 2x	93	87	22
Paternal 4x	88	109	22
Paternal 5x	29	36	5
Paternal 5x+	33	40	2
Paternal 6x	89	115	81

Number of individuals used per cytotype:

	2x	4x	5x	5x+	6x
Maternal	12	53	0	0	20
Paternal	23	45	2	2	19

Appendix 3F: Fasta alignment file of all accessions, accession OF016 and OF101 raw sequence data, chromas files

Appendix 3F: DOI: 10.25403/UPresearchdata.21509226
Alternative link: **https://figshare.com/s/7648875559a814a5033f**

File names: "Appendix3F_Oxalisobliquifolia_Alignment.fas"
"Appendix3F_OF016__4x_AB101_Forward.ab1"
"Appendix3F_OF101__4x_AB101_Reverse.ab1"