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Friends without benefits: Extensive cytotype sympatry and polyploid persistence in an African geophyte

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Abstract

Premise: Polyploidy is a major factor in plant adaptation and speciation. Multiple mechanisms contribute to autopolyploid frequency within populations, but uncertainties remain regarding mechanisms that facilitate polyploid establishment and persistence. Here we aimed to document and predict cytotype distributions of *Oxalis obliquifolia* Steud. ex A. Rich. across Gauteng, South Africa, and test for evidence of possible mechanisms, including morphological, phenological, and reproductive traits, that may potentially facilitate polyploid persistence.

Methods: Over 320 *O. obliquifolia* plants from 25 sites were cytotyped using flow cytometry, and DNA ploidy was confirmed using meiotic chromosome squashes. Cytotypes were mapped and correlations with abiotic variables assessed using ordinations. To assess morphological and phenological associations with cytotype, we grew multiple cytotypes in a common garden, measured phenotypic traits and compared them using linear models and discriminant analyses. Intercytotype reproductive isolation was assessed using crossing experiments, and AMOVAs based on ITS DNA sequences tested for cytogeographic structure.

Results: Six cytotypes were identified, and most sites had multiple cytotypes. Abiotic variables were not predictive of cytotype distribution. A clear gigas effect was present. Differences in flower size and phenology suggested pollinator interactions could play a role in polyploid persistence. Intercytotype crosses produced seed at low frequency. DNA data suggested diploids and polyploids were largely reproductively isolated in situ, and polyploidization events were not frequent enough to explain high cytotype sympatry.

Conclusions: Diploids and polyploids are behaving as separate species, despite little observable niche differentiation and non-zero potential intercytotype seed set. Tests on biotic interactions and intercytotype F1 fitness may provide insights into diploid and polyploid coexistence.

KEYWORDS

autopolyploidy, cytogeography, cytotype sympatry, minority cytotype exclusion, Oxalidaceae, polyploid persistence, whole genome duplication

Polyploidy is an important factor in adaptation and speciation in many plant lineages (Blanc and Wolfe, 2004; Soltis and Soltis, 2009), and recent data suggests that its frequency has been underestimated (Soltis et al., 2007; Parisod et al., 2010; Suda and Herben, 2013; Barker et al., 2016). Polyploidization events can have profound effects on plant physiology and ecology (Levin, 2002; Ramsey and Ramsey, 2014). The generation of duplicate gene copies offers the potential to evolve novel or varied functions that can facilitate changes in the expression of genes in higher-ploidy cytotypes (Adams, 2007; Jiao and Paterson, 2014; Yoo et al., 2014; Saminathan et al., 2015; Coate et al., 2016;

Gallagher et al., 2016). Polyploidization can result in marked changes to phenotype (Levin, 1983; Lumaret, 1988; Bretagnolle et al., 1995; Balao et al., 2011), with immediate consequences for polyploid ecology (Ramsey, 2011; Hahn et al., 2012; Ramsey and Ramsey, 2014; Gallagher et al., 2016), selection (Bretagnole and Thompson, 1996; Jiang et al., 1998; Balao et al., 2011), and responses to environmental conditions (Adams and Wendel, 2005; Duchoslav et al., 2020). All have direct implications for establishment, persistence, and evolutionary fate of higher-ploidy cytotypes.

There is some debate regarding the mechanisms that enable polyploid individuals to first establish and then persist or spread within populations. Newly formed polyploid individuals, by necessity, emerge in an existing diploid parental population, creating a majority cytotype (the parent diploid) and a minority cytotype (the newly emergent polyploid; Levin, 1975). The new polyploid is exposed to minority cytotype exclusion (MCE; Levin, 1975), a frequency-dependent process whereby the minority cytotype is at a reproductive disadvantage through the compounded effects of high frequencies of interploid crosses (since initially only the majority cytotype is available for breeding; Chrtek et al., 2017). If successful intercytotype reproduction is possible, then higher-ploidy cytotypes may be produced by way of a triploid bridge (Burton and Husband, 2001; Yamauchi et al., 2004; Peckert and Chrtek, 2006). Ultimately, a triploid bridge facilitates crosses between tetraploids and triploids, increasing prevalence of unreduced gametes and likelihood of tetraploid (and higher ploidy) establishment within diploid populations (Yahara, 1990; Ramsey and Schemske, 1998; Husband, 2004; Peckert and Chrtek, 2006). However, polyploidization events frequently confer instant reproductive isolation between diploid parents and polyploid offspring (Thompson and Lumaret, 1992; Husband and Schemske, 2000; Husband and Sabara, 2004), often manifesting as a triploid block (Bretagnolle and Thompson, 1995; Felber and Bever, 1997; Köhler et al., 2010) Minority cytotype exclusion poses a major obstacle to polyploid establishment and long-term persistence (Husband, 2000; Ramsey and Schemske, 2002; Otto, 2007; Fowler and Levin, 2016), and polyploids are likely to become established through mechanisms that mitigate the constraints of MCE (Stebbins, 1950).

Therefore, other mechanisms may be important for the establishment of polyploids. Polyploids may achieve higher levels of fitness through increased potential for self-fertilization and clonal reproduction (Levin, 1975; Rodríguez, 1996; Nakayama et al., 2002; Mable, 2004; Yamauchi et al., 2004; Rausch and Morgan, 2005; Hörandl and Hojsgaard, 2012; Hojsgaard et al., 2014; Hojsgaard and Hörandl, 2019; Van Drunen and Husband, 2018, 2019; Spoelhof et al., 2020), potentially prolonged lifecycles/iteroparity (Rodríguez, 1996), and perenniality (Gustafsson, 1948; Stebbins, 1950; Rodríguez, 1996; te Beest et al., 2012; Chrtek et al., 2017). Strategies that result in prolonged lifecycles or increased perenniality create the potential for an individual to persist long enough for a compatible mate to arise.

The rate at which polyploidization events occur in a lineage can also impact polyploid persistence via continuous introduction of new polyploids. There is extensive evidence in the literature (for example in Tsigenopoulos et al., 2002; Jang et al., 2018) for polyploids with multiple origins, lending support to this mechanism contributing to polyploid persistence. This evidence suggests that polyploidization events are potentially far more frequent than is apparent based on the number of extant polyploids and on the inferred number of polyploid origin events, particularly in lineages with multiple higher-ploidy cytotypes. New polyploids increase the number 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, polyploidization rates are known to be variable, even in lineages where polyploidy is widespread (for example, Otto and Whitton, 2000).

Polyploid success could also depend on traits that allow them to outcompete or minimize competition with the parent cytotype (Levin, 1975). A competitive advantage may potentially be conferred on polyploids due to direct changes in phenotype and/or morphology associated with increased genome size. One frequent direct effect of polyploidization, known as the gigas effect, is an increase in cell size (Müntzing, 1936; Stebbins, 1971; Masterson, 1994), which has immediate consequences for the physiological traits of the plant that may impact plant ecology. Increased cell size is also often correlated with changes in morphology (te Beest et al., 2012). Polyploids are often 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 et al., 2021) over diploid parents (for example in *Dactylis glomerata*; Maceira et al., 1993).

Alternatively, ecological niche differentiation could minimize competition with parent diploids (Pfennig and Pfennig, 2009), either by spatial (Husband et al., 2013; Karunarathne et al., 2018) or temporal (Pires et al., 2004) differentiation or through resource (such as with available pollinators; Casazza et al., 2017) partitioning between co-occurring cytotypes. Niche differentiation has been observed in many species for aspects of both abiotic (Borrill and Linder, 1971; Lumaret et al., 1987; te Beest et al., 2012; Husband et al., 2013) and biotic niches (Lumaret, 1988; Lumaret and Barrientos, 1990; Muchhala and Potts, 2007; Casazza et al., 2017).

Each of the mechanisms described above can directly impact the ability of polyploids to establish and persist and, therefore, have direct implications for patterns of cytogeography. If polyploidization results in greater competitive ability, we would expect diploids to be displaced by polyploids (Maceira et al., 1993; Castro et al., 2023), resulting in distinct and largely non-overlapping distributions between the two groups. Likewise, competition avoidance by spatial niche differentiation (Lumaret et al., 1987; Sonnleitner et al., 2010;

Karunarathne et al., 2018) would have similar cytogeographic implications. However, other types of ecological niche differentiation, either temporal differentiation (see e.g., Petit et al., 1997; Pires et al., 2004) or resource partitioning, would allow cytotypes to occupy the same geographic niche. We would also expect to find sympatric diploids and polyploids in a species population with very high rates of polyploidization (Fowler and Levin, 2016) or where reproductive isolation between diploids and polyploids has broken down (Husband, 2004; Peckert and Chrtek, 2006; Li et al., 2022).

The aims of this investigation were to document the occurrence of different cytotypes of *Oxalis obliquifolia* (family Oxalidaceae) across Gauteng Province, South Africa, and to assess hypotheses explaining the persistence of polyploids in this species. If we find extensive cytotype sympatry, we expect to see ecological niche differentiation, reduced reproductive isolation, and/or multiple polyploid origin events as potential explanations.

To achieve these aims, four hypotheses were investigated. Firstly, we hypothesized that polyploids will avoid competition with diploids by either occupying different geographic areas or different niches. Secondly, we hypothesized that polyploids are morphologically and phenologically distinct from diploids. Thirdly, we hypothesized that polyploids are reproductively isolated from diploids and that diploid–polyploid crosses do not result in seed set. Lastly, we hypothesized that if there are multiple origin events for polyploids, then variation among ribotypes is more likely to be explained by site as the predictor variable as opposed to cytotype.

MATERIALS AND METHODS

Study species

We tested theories of polyploid persistence using a study system from southern Africa. The genus *Oxalis* L., which has many species within the Greater Cape Floristic Region (GCFR), has substantial intraspecific ploidy variation (Marks, 1956). *Oxalis obtusa* Jacq., a widespread, highly variable species (Salter, 1944), has at least seven distinct cytotypes (Krejčíková et al., 2013a). Similar cytotype distribution patterns have also been found for *Oxalis purpurea* L., with at least five cytotypes identified (Becker et al., 2022). Polyploidy has also been studied in *Oxalis pes-caprae* L. (Castro et al., 2007; Krejčíková et al., 2012), a problematic weed in many other parts of the world, and linked to its invasiveness (Sanz Elorza et al., 2004; Randall, 2012). Unpublished data suggests that this pattern of remarkable ploidy diversity is common throughout *Oxalis* in the GCFR (K. C. Oberlander, personal observations), but nothing is known of the cytogeography of the approximately 30 African *Oxalis* species outside of the GCFR.

Oxalis obliquifolia Steud. ex A. Rich. (Figure 1A), which belongs to a predominantly GCFR clade (Oberlander et al., 2011) and is a close relative of *O. obtusa*, is a perennial, bulbous geophyte with the largest distribution range of all African *Oxalis*. It occurs throughout the eastern, summer-rainfall regions of South Africa (Exell, 1963), northward through eastern Africa to Ethiopia (Raimondo et al., 2009), Eritrea (Edwards et al., 2000), and Sudan (Darbyshire et al., 2015). Vegetatively produced offspring are the result of bulbils produced adventitiously from the underground rhizome, or from the parent bulb itself, and grow to establish clusters of clonal adult plants. It flowers during the rainy months of the austral summer (October to February). The species is tristylous, where successful seed set is governed by pollen transfer between anthers and stigmas of equivalent levels between plants (Barrett, 1990; Krug et al., 2012).

Preliminary data indicate substantial ploidy variability (J. Suda, unpublished data), and the lack of close relatives (such as *O. depressa* Eckl. & Zeyh. and *O. setosa* E. Mey. ex Sond.; Salter, 1944) of *O. obliquifolia* over much of its range implies that polyploids in this species are most likely autopolyploids. These data make this species a particularly promising candidate to test hypotheses pertaining to polyploid evolution in an African context. Despite recent progress (Rice et al., 2019), there is a notable paucity of information regarding ploidy variation in the flora of southern Africa outside the GCFR.

Field sampling

A total of 28 sites across Gauteng Province over an area of approximately 9500 km^2 were selected for sampling based on documented occurrence data on the Global Biodiversity Information Facility (GBIF Secretariat, 2021) and the citizen science website iNaturalist (2021). Fresh leaf material from at least 10 individuals per site, where possible, was harvested for cytotype identification. To avoid sampling individuals of the same genet and to collect individuals across a larger area for each site, individuals were sampled at least 35 m apart, and different flower morphs (long-, middle-, and short-styled) were included whenever possible. Coordinate data (accurate to between 8 m and 12 m) were recorded for all sampled plants 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 1).

Flow cytometry and chromosome counts

Fresh leaf material was stored at 4°C until analysis. Within 3 days of collection, DNA ploidy levels were identified with relative fluorescence intensities of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei using standard flow cytometric techniques (adapted from Krejčíková et al., 2013a). A two-step procedure using buffers Otto I (0.1 M citric acid, 0.5% v/v Tween 20; Otto, 1990) and Otto II (0.4 M $Na_2HPO_4{\cdot}12H_2O$) was used. Leaf tissues were chopped together with an equal amount of an internal reference standard. *Oxalis articulata* was selected as the

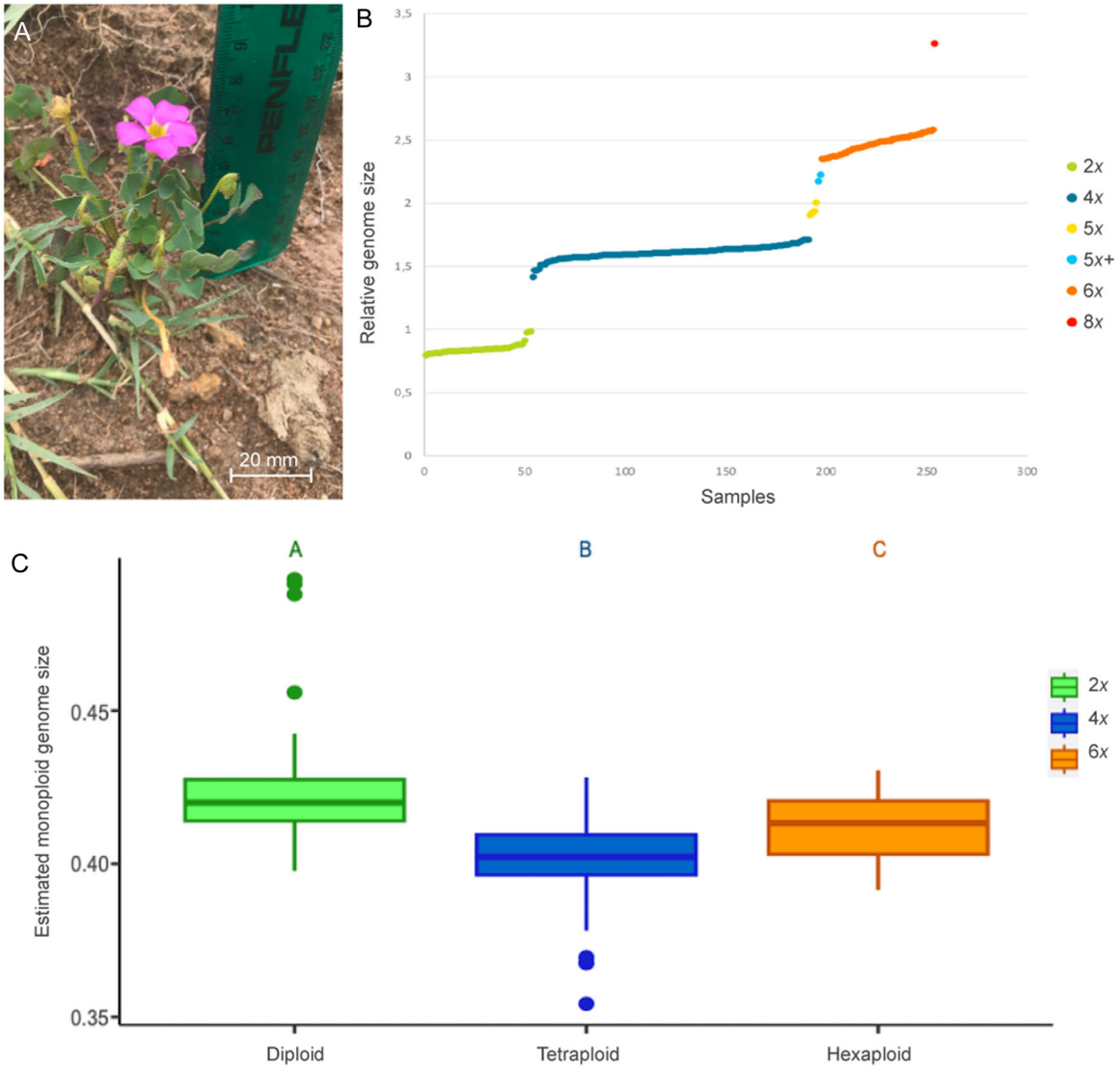

FIGURE 1 (A) *Oxalis obliquifolia* Steud. ex. A. Rich plant. (B) Relative genome sizes of 255 individual *O. obliquifolia* plants, with good CV values (below 5%), collected from across Gauteng Province, South Africa, with six distinct cytotypes identified. (C) Estimated relative monoploid genome sizes of the three major cytotypes. Letters indicate significant differences in size between cytotypes.

internal standard, based on the availability of approximate genome size information (2C-x = 0.91 pg; Vaio et al., 2016). Relative fluorescence of at least 5000 particles were captured using a CyFlow Space cytometer (Sysmex Europe GmbH, Norderstedt, Germany) equipped with a UV laser (352 nm; Doležel et al., 2007) as the light excitation source. Resulting fluorescence histograms were analyzed using FloMax software (version 2.4, Sysmex Partec GmbH, Goerlitz, Germany). Samples were re-analyzed when the coefficient of variation (CV) for any peak was above 5%. If after three separate runs CV values below 5% could not be achieved, peaks that consistently lay within a range of values associated with a particular genome size were assigned to that cytotype. To assess the validity of the flow cytometry results, chromosome numbers for a subset of DNA diploids (20 individuals) and DNA tetraploids (25 individuals) (as determined by flow cytometry) were confirmed using meiotic chromosome squash techniques as described by Windham et al. (2020).

Mapping and data collection

A total of 327 individuals with known cytotypes were mapped using ArcGIS Pro version 10.0 (ESRI, Redlands, CA, USA) across 25 sites, and climate, topography, and underlying geology were extracted for the coordinates for each plant. Mean annual precipitation, minimum temperature of the coldest month (July), and maximum temperature of the hottest month (January) were obtained from the WorldClim 2 data set (version 2.1) at 30 seconds (or approximately 1 km) resolution (Fick and Hijmans, 2017). Slope and northness were calculated from the Shuttle Radar Topography Mission data (SRTM; Jarvis et al., 2008) at 30 second resolution using

ArcGIS Pro. Geological data were obtained from the chronostratigraphic map by Keyser (1997). Microclimatic variables relating to sun exposure (sun or shade; obtained using field observations) and soil texture were also included. Soil samples (50 mL) were collected and thoroughly air-dried, then stored in airtight containers at −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. Soil texture was characterized by separating the coarse and soil fractions (using a 2-mm sieve) and calculating the percentage of coarse material in the total dry mass. The soil texture was then further characterized using the ribbon method, as described by the Natural Resources Conservation Service, United States Department of Agriculture and based on the methods described by Thien (1979) (Appendix S1).

Common garden experiment

A total of 98 individuals from 12 different sites (6–10/site) and comprising all three major cytotypes (diploids from four sites, tetraploids from 10 sites and hexaploids from 2 sites, which included individuals from both single-ploidy and mixed-ploidy sites) were chosen for common garden experiments. Ploidy levels were confirmed using flow cytometry as described above. Bulbs were planted at 5 cm below the soil surface in 13-cm-diameter plastic pots containing a homogenized mixture of sand and potting soil. Plants acclimated and entered dormancy for a full season (over winter, from end of March 2021 to end of August 2021). For the subsequent growing season (334 days, beginning 28 August 2021), plants were watered with 110 mL every second day until plants fully senesced and re-entered dormancy. Plants were grown outside in full sun and randomly shuffled once a week to minimize the effect of possible microclimatic variations.

Morphological traits of the common garden plants were measured at the peak of the growing season (end of January 2022) and 2 months after the emergence of each plant to account for the possible effect of emergence time on individual morphology. 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 on plants were measured using callipers and a ruler to an accuracy of 0.5 mm. The first flower to open (from the date of measuring) and the largest mature leaf were always measured. Other quantitative data included the bract position (measured from the base of the peduncle), number of leaves (excluding those that had completely senesced or detached) and number of inflorescences (including fruiting and immature inflorescences). Shape characters were recorded as ratios between size measurements of principal organs and were log-transformed before 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. Two qualitative traits (flower color and color of the abaxial leaf surface) were also captured (Appendix S2), but these did not explain any variability in the data in preliminary analyses and were discarded.

Phenological data were captured daily between 07:00 hours and 12:00 hours over the growing season. Phenological events included date of emergence, date of first anthesis, date of last flower senescence, and the date of final senescence (when the last leaf turned yellow). These dates were used to generate count data (measured as the number of days since initial watering) for the vegetative phenology and flowering phenology. For flowering phenology, only a subset of plants (nine diploids, 14 tetraploids, and eight hexaploids) could be included because the date of last flower senescence was only included as a trait later in the study.

Cytotype crossing experiment

Reproductive isolation between cytotypes was measured using a hand-pollination experiment (1140 crosses: 432 used maternal diploids, 499 used maternal tetraploids, and 209 used maternal hexaploids) in controlled conditions using the procedure of du Preez et al. (2018). Pollination treatments included (1) self-pollination (stigma pollinated with an anther from the same flower) (2) intraploid pollination (stigma pollinated using an anther from a compatible flower morph of the same cytotype), (3) interploid pollination (stigma pollinated using an anther from a compatible flower morph from a different cytotype). Crosses included all combinations between the type of cross performed (self-pollinated, intraploid or interploid) and the maternal cytotype (diploid, tetraploid, or hexaploid). Pollinations were conducted between 07:00 hours and 12:00 hours daily from September 2020 to March 2021 using virgin flowers. All crosses were conducted between compatible stigmas and anthers of the same level (long-, mid- and short-styled; du Preez et al., 2018) to account for tristyly. Each flower was then emasculated using alcohol-sterilized forceps. Subsequent pollinator-vectored pollen was excluded by removing petals and enclosing the flower in an empty teabag, which also retained the explosively ejected seeds after fruit dehiscence. Unused open flowers were removed daily to avoid confusion with subsequent virgin flowers. Peduncles that withered and detached within 2 weeks (on average) from pollination were considered unsuccessful fertilizations. For successful crosses, intact teabags were inspected each day for fruit dehiscence and seed release, and seeds from each cross were counted to determine seed set.

DNA extraction, sequencing and analysis

To test for relatedness between individuals and test for the possible number of polyploid origin events, at least two representatives of each different cytotype (diploids,

tetraploids and hexaploids) from 14 sites were studied using barcoding techniques. Fresh leaf material from 86 individuals was collected in silica gel. DNA was extracted as described by Oberlander et al. (2004) using a modified 2X CTAB method (Doyle and Doyle, 1987). Extracted DNA quality and quantity were assessed using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The internal transcribed spacer (ITS) region (Sun et al., 1994) was used as the target marker. The reaction mixture for the PCRs contained 12.5 µL of Ampliqon *Taq* MasterMix (Ampliqon, Odense, Denmark), 8 µL distilled water, 0.5 µL of 50 mM $MgCl_2$, 1 µL of 10 µM each ITS primer (forward and reverse), and 2 µL of template DNA. PCR thermocycling conditions were initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 5 min. Standard post-PCR clean-up and dideoxy-terminated Sanger sequencing of the PCR products were done by the Central Analytical Facility at the University of Stellenbosch. Chromas software version 2.6.6 (Technelysium, Brisbane, Australia) was used for chromatogram base-calling verification, and BioEdit (version 7.2.5; Hall, 1999) was used for assembling contigs and manual DNA alignment. Nucleotide polymorphisms were coded using standard IUPAC degenerative coding. Sequences were screened for potential contamination using BLAST searches against GenBank (NCBI; https://www.ncbi.nlm.nih.gov/genbank/) submissions.

Statistical analyses

Unless otherwise indicated, all statistical analyses were performed in the R statistical environment (version 4.2.0; R Core Team, 2022). Differences in estimated monoploid genome sizes between the three majority cytotypes, across all populations, were assessed with an ANOVA followed by Tukey's test using the multcomp package (Hothorn et al., 2008). To assess whether the sampling was sufficient to detect the actual cytotype diversity across the study area, we generated a cytotype accumulation curve and calculated a jacknife1 index value was calculated using the vegan package (Oksanen et al., 2022).

To assess abiotic conditions as predictor variables (10 variables in total) for specific cytotypes, we used a multiple factor analysis (MFA) in the FactoMineR package (Lê et al., 2008). Abiotic variables were assessed for autocorrelation using Pearson's correlation coefficient (R Core Team, 2022), and a single random variable from any correlated pair with an $|r|$ value of more than 0.7 was retained for analysis. Elevation was highly correlated with both maximum ($|r|$= 0.94) and minimum temperature ($|r|$= 0.72), and so only elevation was retained for analysis. Statistical support for abiotic variable associations with cytotypes and sites were then determined using Gower's distance (Gower, 1971) and a PERMANOVA using the vegan package (Oksanen et al., 2022).

Morphological traits were also assessed for autocorrelation as above (Appendix S3). Univariate analyses testing morphological differences between cytotypes were conducted using the glm() function with the stats package in base R (GLM; R Core Team, 2022). Data normality for continuous variables was assessed using a Shapiro-Wilk test (Royston, 1982) with subsequent Box-Cox tests using the boxcox function (Box and Cox, 1964; Venables and Ripley, 2002) for best-fit transformations. The fit of each model was assessed by comparing Q-Q plots of residuals, AIC values, and residual deviance values. Negative binomial distributions were used for count data. Ratios were modelled using a quasi-Poisson distribution, and in some cases, the data were log-transformed where these resulted in improved model fit (see Appendix S4). Post hoc tests were done as above. All univariate *P*-values were adjusted using Benjamini and Hochberg (1995) correction for multiple comparisons (R Core Team, 2022).

Linear discriminant analysis (LDA; Venables and Ripley, 2002) was used to assess multivariate differences in quantitative morphological traits between different cytotypes using the FactoMineR (Lê et al., 2008) and MASS (Venables and Ripley, 2002) packages. Initially, two qualitative traits (flower color and abaxial leaf surface color) were assessed using a factor analysis of mixed data with FactoMineR (Lê et al., 2008), but were found to be uninformative and were subsequently removed. To assess the relationship between polyploidy and phenology, we used GLMs with Poisson distributions (R Core Team, 2022) and with multiple test corrections and post hoc tests as described above.

The effect of different types of crosses (self-pollination, intraploid pollination, interploid pollination) and its interaction with maternal cytotype on seed set as a proxy for the presence of possible prezygotic barriers to reproduction. A hurdle model was used to accommodate the zero-inflated distribution of the seed-set data using the R package pscl (Jackman, 2020). A negative binomial distribution was used for the seed-set count data (seed set > zero), and a binomial distribution for the zero (successful/unsuccessful cross) count data. A post hoc Tukey test was performed using the emmeans package (Lenth, 2023).

To test the hypothesis that individuals were more closely related by cytotype than by site, two different approaches were followed on the ITS data set (Appendix 2). First, multiple tree or tree-like methods were used to visualize the similarity/relatedness between individual ribotypes. Hierarchical clustering based on molecular distance/similarity was used to construct a dendrogram. A Bayesian consensus tree with posterior probabilities was constructed with MrBayes software (version 3.2.2; parameters: nst = 6, rates = gamma; Ronquist et al., 2012) using the CIPRES online portal (Miller et al., 2010) and visualized using FigTree (version 1.4.4; Rambaut, 2018).

Additionally, a parsimony tree with bootstrap support values was constructed using PAUP* software (Swofford, 1991; also using CIPRES). Secondly, an AMOVA (Meirmans and Liu, 2018) was used to formally test population structure against cytotype and site using Arlequin (version 3.5.2.2; Excoffier and Lischer, 2010). First, ribotypes were grouped by site then cytotype, which allows for the existence of distinct cytotype populations within individual

sites, and second, by cytotype then site to test which variable order gave greater explanatory power.

RESULTS

Cytotype identification

A total of 320 specimens of *O. obliquifolia* were sampled from the 25 sites across Gauteng Province (Figure 1B). Of those, 255 individuals had CVs below 5% and were used to construct and identify cytotype (i.e., DNA ploidy) categories. The mean CV for sample (G1) fluorescence peaks was 4.19% (range 2.38–4.99%). Six cytotypes were identified (Table 1), including diploids (2*x*; relative genome size = 0.849), tetraploids (4*x*; relative genome size = 1.621), pentaploids (5*x*; relative genome size = 1.944), hexaploids (6*x*; relative genome size = 2.514) and octoploids (8*x*; relative genome size = 3.266), and possibly an instance of aneuploidy (5*x*+; relative genome size = 2.197).

The estimated monoploid genome sizes for the three majority cytotypes did not show the expected additive effect of recent autopolyploids (Figure 1C). All three cytotype monoploid genome sizes differed significantly from one another (Table 2), with hexaploids (mean ± SD: $x = 0.411 \pm 0.011$) intermediate between diploids ($x = 0.424 \pm 0.020$) and tetraploids ($x = 0.402 \pm 0.012$).

Meiotic chromosome squashes (Appendix S5) from both diploids (five individuals from different populations) and tetraploids (five individuals from different populations) confirmed DNA ploidies. Karyotypes for pentaploids, hexaploids, and octoploids could not be confirmed. Chromosome counts were determined to be $2x = 14$ for diploids and $4x = 28$ for tetraploids, with a base chromosome number of $x = 7$. Chromosomes were mostly metacentric to submetacentric (Appendix S5).

Cytotype mapping

Over half (15 of 25) of the sampled sites had mixed-ploidy populations, with remaining sites being either uniformly diploid (two sites), tetraploid (seven sites) or hexaploid (one site) (Figure 2). These three cytotypes comprised the largest portion of individuals encountered. Tetraploids were the most common cytotype (54%).

The MFA showed substantial overlap between cytotype clusters (Figure 2D), with tetraploids co-occurring with both diploids and hexaploids. Total explanatory power of the MFA for the first two axes was very low, accounting for only 19.98% of the variation. Furthermore, only after reaching dimension eight (of 34 dimensions) did the cumulative percentage of variation reach 50%, indicating that abiotic variables do not contribute to determining cytotype distribution patterns in this system at this spatial scale. Notably, site, and not cytotype was most strongly associated with the variation accounted for by dimensions one and two (Appendix S6). Individual clusters suggest local conditions may be more informative than the broad-scale variables considered here. Although abiotic differences between both cytotypes and sites were statistically significant (Table 3), site as the response variable had a far greater sum of squares value (21.858 vs. 3.169), indicating it accounted for most of the variation.

The cytotype accumulation suggested that the encountered number of cytotypes (six cytotypes in total)

TABLE 2 Results of ANOVA to assess differences in estimated monoploid genome sizes associated with *Oxalis obliquifolia* majority cytotypes, and providing R^2 values, *F* statistic and residual SE.

Ploidy	Estimate	SE	*t*	*P*
Diploid (*2x*, intercept)	0.424348	0.001904	222.816	<2e-16*
Tetraploid (*4x*)	−0.022119	0.002241	−9.872	<2e-16*
Hexaploid (*6x*)	−0.012892	0.002657	−4.852	2.18e-06*
Residual SE: 0.01386, df = 244				
Multiple R^2: 0.2899				
Adjusted R^2: 0.2841				
$F = 49.8$, df = 2, 244				<2.2e-16*

*Significant P-values.

TABLE 1 Results of flow cytometric analysis of *Oxalis obliquifolia* leaf samples from each individual plant, indicating ploidy levels, relative genome sizes based of fluorescence histograms, and estimated monoploid genome sizes.

Ploidy	Relative genome size (mean ± SD)[a]	Estimated monoploid genome size (mean ± SD)[b]	*N*	No. of sites encountered
2x	0.849 ± 0.039	0.424 ± 0.020	53	10
4x	1.621 ± 0.048	0.402 ± 0.012	137	21
5x	1.944 ± 0.043	—	4	3
5x+	2.197 ± 0.037	—	2	1
6x	2.514 ± 0.068	0.411 ± 0.011	55	9
8x	3.266	—	1	1

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

[b]Calculated by dividing the relative genome size by the corresponding ploidy level, for the three majority cytotypes.

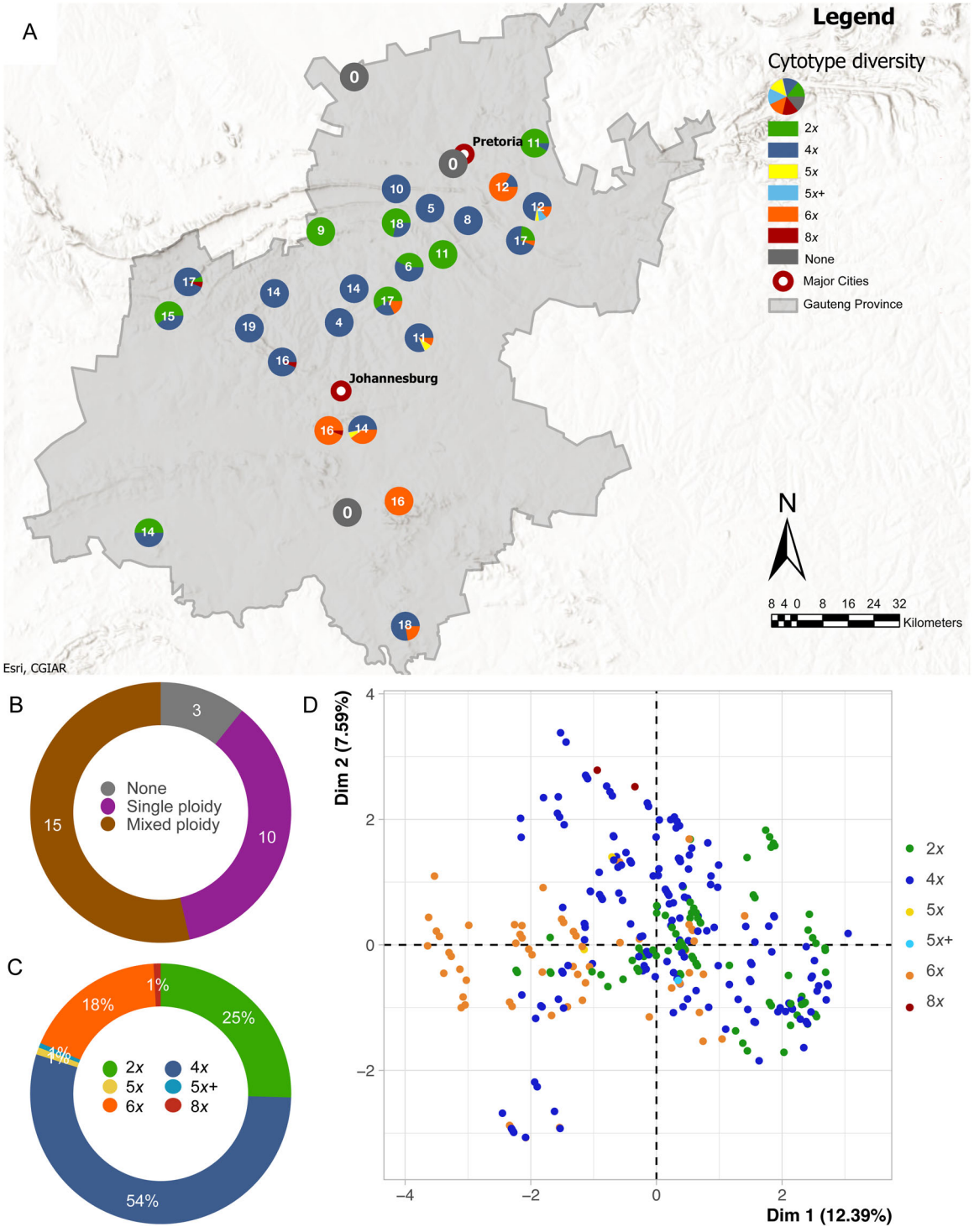

FIGURE 2 (A) Cytogeography of *Oxalis obliquifolia* across Gauteng Province, with the proportion of different cytotypes identified at each locality (grey dots: sites where no plants were found). Numbers indicate the number of individuals samples at each site. (B) Proportion of sites with no *O. obliquifolia*, only one cytotype or mixed cytotype. (C) Total proportion of cytotypes encountered across Gauteng Province. (D) Multiple factor analysis (MFA) based on 10 abiotic environmental variables as predictors of cytotype distribution of *Oxalis obliquifolia* across Gauteng Province. Dimensions 1 and 2 only accounting for a cumulative 19.98% of the variation observed. Colors grouped by cytotype.

is close to the real number of cytotypes present in the sampled area and is supported by a jacknife1 value of 6.96, based on the rate of rare cytotype occurrence across the study area.

Morphological traits

Subtle differences were observed when comparing the morphological data (captured at the peak of the growing

TABLE 3 Results of PerMANOVA analysis testing the strength of associations of abiotic variables, as predictors of cytotype distribution, against both individual sites and against cytotype groups.

Groups	df	SS	R^2	F	P
Cytotype	5	3.169	0.09840	27.0309	1.0×10^{-3}*
Site	24	21.858	0.67869	38.8416	1.0×10^{-3}*
Cytotype × Site	16	0.590	0.01833	1.5735	1.0×10^{-3}*
Residual	281	6.589	0.20458		
Total	326	32.206	1.00000		

*Significant P-values.

season, and at 2 months since emergence; Appendix S7), but both data sets of measurements yielded very similar results in terms of the types of traits (size-related) that were most useful in explaining the variability observed between cytotype clusters. Consequently, we only present the results from the data captured at 2 months after emergence of each plant.

The GLMs showed distinct associations between cytotype and nine of the 16 morphological traits (Table 4), with significant differences observed between diploids and at least one of the higher ploidy cytotypes. The GLM results included significant differences in four of the five foliar traits and five of the 11 floral traits, including many size-related traits (Table 4). Of shape-related characters, middle leaflet shape, petal shape, and the position of bracts on the peduncle differed. Diploids had smaller leaflets and higher numbers of leaves than higher ploidy cytotypes. Diploids were significantly smaller than tetraploids, but not hexaploids, for petiole length and the ratio of middle leaflet length to middle leaflet width. Regarding floral characters, diploids had more inflorescences, shorter peduncles, bracts positioned higher on the peduncle, shorter and narrower (i.e., smaller) sepals, and narrower petals. Petal shape also differed significantly between diploids and hexaploids, with hexaploids having a larger petal length to petal width ratio. Despite these general trends, there was substantial overlap for all these traits, (Figure 3A), resulting in no trait being a truly reliable predictor of cytotype.

The LDA results (Figure 3B) showed 88.26% separation of cytotypes on the first linear discriminant axis (LD1) and 11.74% on the second linear discriminant axis (LD2), with an overall LDA model prediction accuracy of 72.22%. Based on the coefficients of linear discriminants the predictor variables that are most influential in creating the decision rule of the LDA model 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. These results again suggest that size-related traits (both floral and foliar) are most informative in distinguishing between cytotype clusters.

Phenological traits

Significant phenological differences were observed between cytotypes (Table 5, Figure 4). Diploids emerged earlier (an average of 66.7 days after initial watering; Figure 4A and 4B) than tetraploids (71.0 days average) and hexaploids (79.3 days average). Additionally, diploids had a longer growing season (average of 226.3 days from emergence to final senescence), than tetraploids (216.3 days) and hexaploids (200.8 days; Figure 4A, B). Differences in flowering phenology were notably more distinct than differences in other phenological variables. Diploids began flowering earlier (113.7 days from first watering; Figure 4C, D) than tetraploids (126.6 days) and hexaploids (130.5 days). Diploids had a substantially longer flowering period (137.4 days; Figure 4C, D) than either tetraploids (102.9 days) or hexaploids (116.4 days). Both major polyploid cytotypes had a narrower peak flowering season, that occurred near the beginning of the longer diploid flowering season (Figure 4C, D). The peak flowering season (when all plants were flowering) for diploids occurred between 120 to 166 days after first watering. Peak flowering for tetraploids and hexaploids occurred between 86 to 119 days and 61 to 136 days since first watering, respectively.

Success rate of crosses

Self-pollination success rates were very low in all cytotypes (Figure 5A; Appendix S8), and no significant difference in self-pollination success rates between different maternal cytotypes was observed (Table 6, Figure 5A). Success rates of selfing and interploid cross involving maternal diploids (approximately 5.9% successful) were not significantly different (Figure 5A). Regarding successful interploidy crosses with maternal diploids, 35.7% involved crosses with paternal tetraploids and 64.3% involved crosses with paternal hexaploids. Nor did success rates differ significantly between the selfed and interploid crosses with maternal hexaploids (15.7%). Regarding successful interploidy crosses with maternal hexaploids, 12.5% involved crosses with paternal diploids and 87.5% involved crosses with paternal tetraploids. However, success rates differed significantly between self-pollination and interploid crosses with maternal tetraploids (16.2%). In other words, maternal tetraploids were able to successfully cross with other cytotypes and produce seed (Figure 5A). Regarding successful interploidy crosses with maternal tetraploids, 13.3% involved crosses with paternal diploids, and 86.7% involved crosses with paternal hexaploids. As expected, success rates of intraploid crosses were substantially higher than those of self-pollination and interploid across all maternal cytotypes. Diploids had an intraploid success rate of about 64.1%, tetraploids 57.8%, and hexaploids the highest of approximately 80.0%.

Seed set among successful crosses

Seed set (for successful crosses) was higher in interploid crosses than seed set for successful self-pollinations, which

TABLE 4 Cytotype morphological quantitative traits (unit), means (SD), and adjusted *P*-values of GLM analyses, using Benjamini-Hochberg corrections (2*x*–4*x*, 2*x*–6*x*), and Tukey post hoc test results (2*x*–4*x*, 2*x*–6*x*, 4*x*–6*x*; also indicated in Figure 3A).

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

TABLE 4 (Continued)

Trait	2*x*	4*x*	6*x*	Adjusted *P* 2*x*–4*x* 2*x*–6*x*	Tukey 2*x*–4*x* 2*x*–6*x* 4*x*–6*x*
12. Ratio petal length to width	1.91 (0.26)	1.85 (0.21)	1.75 (0.22)	2.95×10^{-1} 3.67×10^{-2}*	5.40×10^{-1} 5.66×10^{-2} 2.14×10^{-1}
13. Ratio sepal length to width	2.42 (0.52)	2.58 (0.51)	2.41 (0.45)	2.61×10^{-1} 8.72×10^{-1}	3.53×10^{-1} 9.85×10^{-1} 5.27×10^{-1}
14. Ratio peduncle length to bract position	1.09 (0.04)	1.12 (0.11)	1.14 (0.07)	5.26×10^{-2} 5.26×10^{-2}	1.05×10^{-1} 1.20×10^{-1} 9.16×10^{-1}
15. Number of inflorescences	8.6 (4.8)	5.5 (2.9)	6.5 (3.0)	4.60×10^{-5}* 3.34×10^{-2}*	$<1.0 \times 10^{-3}$* 8.33×10^{-2} 3.96×10^{-1}
16. Difference in peduncle and petiole length (mm)	5.9 (20.5)	6.3 (23.7)	19.9 (22.0)	9.27×10^{-1} 1.37×10^{-1}	9.95×10^{-1} 1.05×10^{-1} 6.18×10^{-2}

*Significant P-values based on GLM results.

is expected in a self-incompatible species; however, as expected, the highest seed set was produced in intraploid crosses, a pattern that was consistent across all maternal cytotypes (Figure 5B). Regarding crosses with maternal diploids, there was a significant difference between the number of seeds produced by interploid (mean of 5.5 seeds) and intraploid (mean of 19.0 seeds) crosses (Table 6, Figure 5B). The only successful self-pollination of a maternal diploid plant produced two seeds. The single successful self-pollination cross with a maternal hexaploid produced one seed. In the four successful self-pollination crosses with maternal tetraploids, a mean of 2.5 seeds were produced. Self-pollination seed set was not significantly different between maternal cytotypes (Figure 5B). Mean seed set of interploid crosses with maternal tetraploids was 4.0 seeds, compared to a mean of 9.5 seeds for interploid crosses with maternal hexaploids. Mean seed set for intraploid crosses in maternal tetraploids and maternal hexaploids was 11.0 and 13.0 seeds, respectively. Finally, diploids produced the highest mean seed set for intraploid crosses (mean = 19.0 seeds) compared with both tetraploids (mean = 11.0 seeds) and hexaploids (mean = 13.0 seeds), although these differences were not significant.

Gene flow and polyploidization frequency

The ITS sequence alignment was 790 bp in length, with 15 variable sites among the 82 individuals included. Within these variable sites, 13 were parsimony-informative, with two singletons. Dendrograms constructed from ITS data separated diploids and polyploids into two discrete clusters (Figure 6), with the intervening branch having a bootstrap value of 94% and posterior probability of 1.00. There was only one exception of a single diploid individual with a polyploid ribotype (accession OF016), found at a mixed diploid–tetraploid site. Individuals clustered according to ploidy (diploids vs polyploid) and not site. Within the polyploid cluster, tetraploids and hexaploids showed very little structure.

When grouped by site first (Table 7), ITS variation in the AMOVA was largely explained by differences between cytotypes within sites, rather than differences observed between sites. Furthermore, little difference was observed within cytotypes, across individual sites. When ribotypes were grouped according to cytotype first, almost all the variation was observed between cytotypes and not site (Table 7).

DISCUSSION

There is a remarkably high level of cytotype diversity in this system, with a substantial degree of cytotype sympatry. Estimates of monoploid genome sizes suggest that genome downsizing may have occurred, which implies that polyploidization did not occur recently and that other mechanisms have facilitated polyploid persistence and cytotype sympatry. Cytotypes appear to share the same spatial niche,

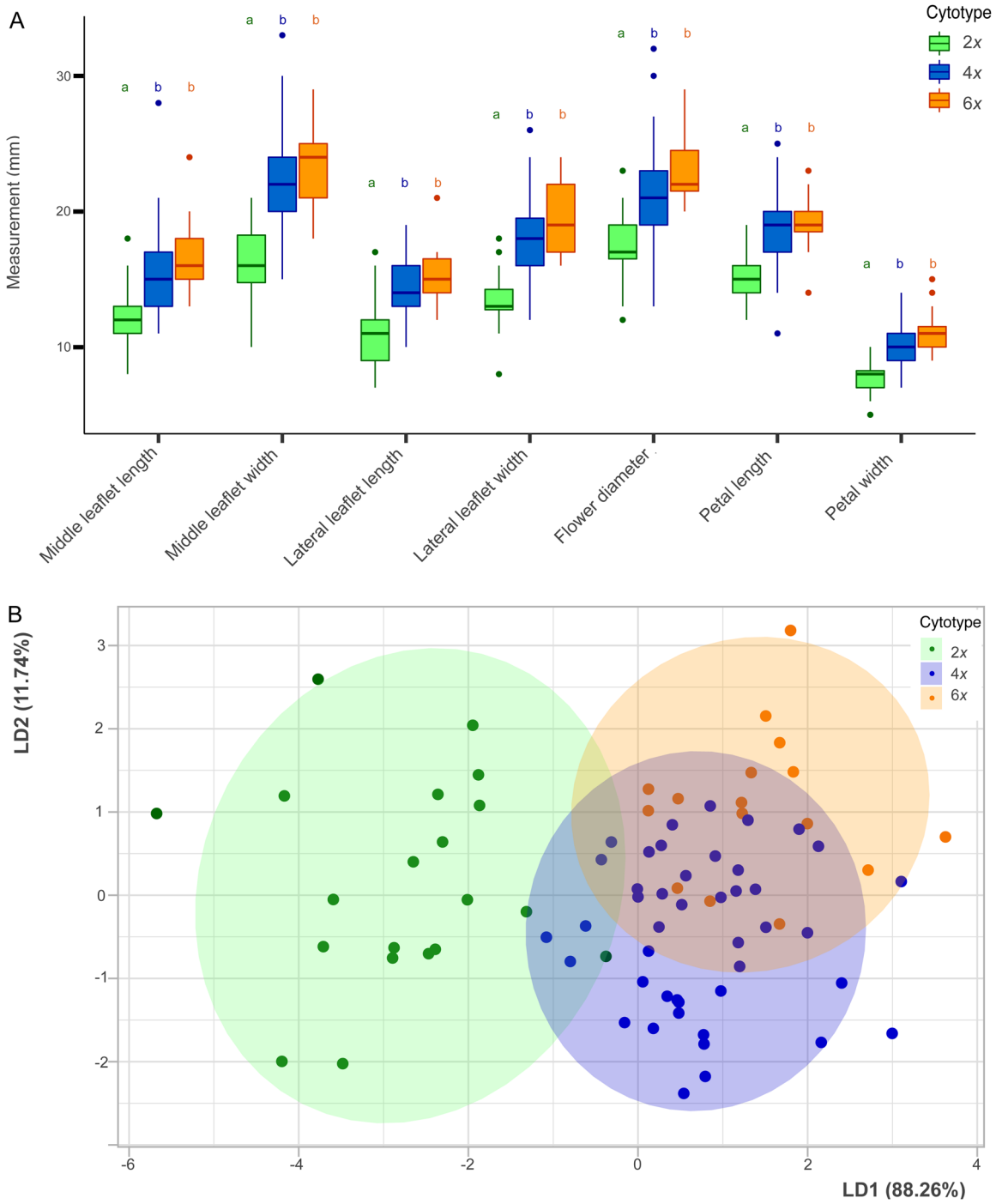

FIGURE 3 (A) Boxplots of selected foliar and floral size-related traits measured for different cytotypes included in a common garden experiment and captured during the height of the growing season. Letters above plots denote statistically significant differences. (B) Linear discriminant analysis constructed using all 23 quantitative morphological traits as predictors of cytotype, showing clear separation of diploid vs polyploid clusters, with 95% confidence ellipses indicated for each group.

but there is some evidence of potential temporal niche differentiation. Polyploids tend to flower earlier than diploids. Additionally, polyploids are also larger than their diploid counterparts, with regards to both floral and foliar traits. Furthermore, evidence suggests that there is asymmetrical reproductive isolation in the wild between diploids and polyploids.

Intraspecific ploidy variation

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). The majority have a base

TABLE 5 Phenological data (in days), means (SD), and adjusted *P*-values of generalized linear model analyses after Benjamini and Hochberg corrections (2*x*–4*x*, 2*x*–6*x*), and for Tukey post-hoc tests (2*x*–4*x*, 2*x*–6*x*, 4*x*–6*x*; also indicated in Figure 4B and 4D).

Phenological trait	2*x*	4*x*	6*x*	Adjusted *P* 2*x*–4*x* 2*x*–6*x*	Tukey 2*x*–4*x* 2*x*–6*x* 4*x*–6*x*
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} $<1.0 \times 10^{-4}$* $<1.0 \times 10^{-4}$*
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 aboveground (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}* $<1.0 \times 10^{-4}$* $<1.0 \times 10^{-4}$*
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}*	$<1.0 \times 10^{-4}$* $<1.0 \times 10^{-4}$* 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}*	$<1.0 \times 10^{-4}$* $<1.0 \times 10^{-4}$* 9.20×10^{-3}*

*Significant P-values based on GLM results.

number of $x = 7$. These chromosomes exhibit a range of diverse morphologies regarding centromere placement, including metacentric, submetacentric, telocentric and acrocentric (de Azkue and Martínez, 1983, 1984, 1988, 1990; de Azkue, 2000).

We demonstrate a base chromosome number of $x = 7$ for *O. obliquifolia*, which agrees with expectations based on chromosome counts for close relatives, including *O. obtusa*, which also has a base chromosome number of $x = 7$ (Krejčíková et al., 2013a). 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 Martínez, 1990; de Azkue, 2000; Dreyer and Johnson, 2000; Sato et al., 2008; Vaio et al., 2013; Krejčíková et al., 2013a; Moura et al., 2020).

Gauteng populations of *O. obliquifolia* harbor impressive cytotype diversity. Five distinct cytotypes (2*x*, 4*x*, 5*x*, 6*x*, and 8*x*) and one possible case of aneuploidy (5*x*+) were encountered. This cytotype diversity is comparable to that encountered across the entire distribution of closely related *Oxalis* (Krejčíková et al., 2013a, 2013b) and is exceptionally high when compared with cytotype distributions of other species at larger geographic scales (such as Marhold et al., 2010; Frajman et al., 2015). The very close (but not identical) estimates of relative monoploid genome sizes for diploids, tetraploids, and hexaploids suggest that these higher-ploidy cytotypes are most likely autopolyploids. Autopolyploidy also supports previous suggestions of very limited to no hybridization events in the southern African *Oxalis* lineage (Salter, 1944). The slightly smaller monoploid size associated with polyploids is typical of genome downsizing, suggesting that the origin of this polyploid lineage is not a recent event (Leitch and Bennett, 2004; Wang et al., 2021). Although less likely in our view, such close monoploid estimates may indicate hybridization between species that have very similar genome sizes (allopolyploidy), as observed in polyploid complexes of other species (such as Pellicer et al., 2012). It is possible that an extinct diploid ancestor (e.g., Li et al., 2014) may have contributed to an allopolyploid *O. obliquifolia*, but we have no evidence of such an ancestor. However, there are no close relatives of *O. obliquifolia* that co-occur across most of its distribution. Additionally, the intermediate monoploid size of hexaploids could indicate hybridization between diploids and tetraploids. However, our crossing experiment results and ITS analyses suggest that this is unlikely. Instead, the slightly larger size of hexaploids may indicate that the formation of hexaploids is a more recent event and/or that genome downsizing has progressed more slowly in hexaploids.

Cytogeography in Gauteng Province

Oxalis obliquifolia has a complex cytotype distribution across Gauteng, with a remarkable degree of sympatry (>50% of sampled sites). There are no distinct patterns of cytotype distribution, an observation supported by the MFA. It is possible that distinct cytotype distribution patterns might be observed across larger parts of the distribution range (e.g., Hijmans et al., 2007; Manzaneda et al., 2012; Sutherland and

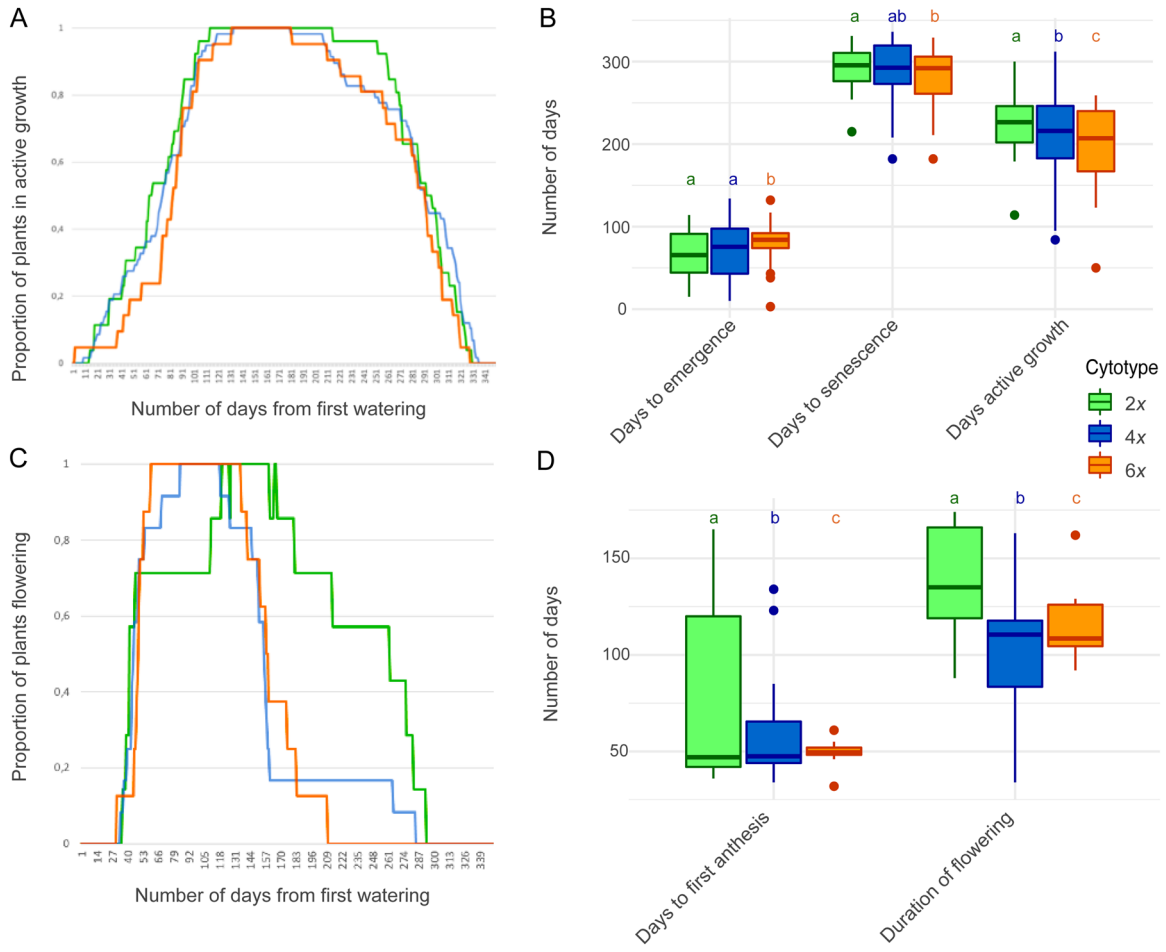

FIGURE 4 Results of common garden experiment. (A) Proportion of actively growing *Oxalis obliquifolia* diploids, tetraploids, and hexaploids. (B) Boxplots of vegetative growth phenology measured for different cytotypes. (C) Proportion of flowering *O. obliquifolia* individuals, including diploids, tetraploids, and hexaploids. (D) Boxplots of flower phenology measured for different cytotypes. Different letters above plots denote statistically significant differences in values of traits associated with different cytotypes.

Galloway, 2018; Semple et al., 2021), given the small sampled area. Cytotype distribution patterns are the result of complex interactions among cytotypes and between cytotypes and other species (Stebbins, 1985; Thompson and Lumaret, 1992; Buggs and Pannell, 2007; Martin and Husband, 2009; Husband et al., 2013; Kolář et al., 2009, 2017). These can range from completely discrete cytogeographic patterns (allopatry), adjacent to slightly overlapping distributions (parapatric), to complete diploid and polyploid sympatry (Husband and Schemske, 1998; Trávníček et al., 2011).

This study supports arguments for site-intensive sampling to allow more accurate assessments of within-population cytotype variation (e.g., Pierson et al., 2012; Castro et al., 2018; Rejlová et al., 2019). Mixed-cytotype populations may be underrepresented in many existing studies that have limited numbers of samples per site. It is notable that there are instances where studies on ecological (often climatic) differentiation between polyploids and diploids have not always supported habitat segregation (Godsoe et al., 2013; Glennon et al., 2014).

There were no definite patterns in the geographic distribution of cytotypes in the study area. Instead, there was wide co-existence of different cytotypes: eight different cytotype combinations were encountered. Mixed-cytotype populations are well documented in many species (Burton and Husband, 1999; Weiss et al., 2002; Španiel et al., 2008). They have been used to indicate contact zones between broader distinct distributions of single-ploidy populations (e.g., Husband and Schemske, 1998; Mráz et al., 2012; Sabara et al., 2013; Zozomová-Lihová et al., 2015). These types of systems are often encountered in the northern hemisphere, which are subject to glacial and interglacial population expansion and refugial dynamics (Majure et al., 2012; Sutherland and Galloway, 2018) and therefore are often characterized as non-equilibrium systems. This pattern of smaller contact zones between distributions is in contrast with the extensive and widespread cytotype sympatry observed in this system. It remains to be seen whether the extensive cytotype sympatry in this system may be part of a much larger contact zone between different cytotype populations and whether this system is characterized by equilibrium dynamics. Future work should focus on a larger study area to test these possibilities. However, the high degree

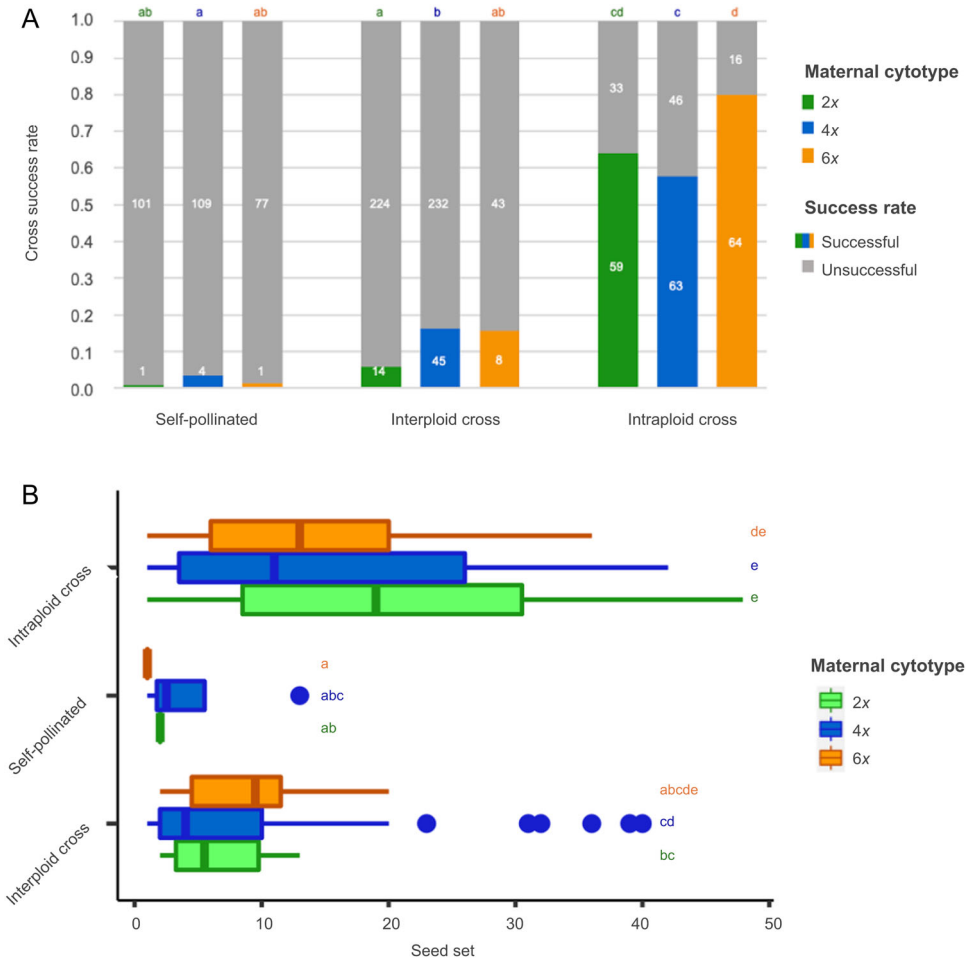

FIGURE 5 (A) Proportion of successful (1 or more seeds) vs unsuccessful (0 seeds) crosses for each combination of maternal cytotype and type of cross. Internal numbers indicate the count for successful or unsuccessful crosses for each type. Different letters above plots denote statistically significant differences. (B) Seed set for successful crosses for each combination of maternal cytotype and type of cross (intraploid, interploid, and self-pollinated). Different letters to the right of the boxplots denote statistically significant differences.

of cytotype sympatry (up to three at a single locality) suggest a highly complex system, where abiotic variables are not the primary drivers of cytotype distribution patterns.

Studies have also shown that polyploidization has been directly linked to range expansion (McIntyre, 2012; Voss et al., 2012; Maguilla et al., 2021) and can even result in increased invasiveness (te Beest et al., 2012). It is notable that *O. obliquifolia* is deeply embedded in a mostly GCFR lineage, and its huge pan-African distribution range is highly unusual compared to the distribution ranges of close relatives. It is possible that polyploidy has played a role in the "invasion" of the African continent by this species during its evolutionary history. Further sampling, especially from the central and northern portions of the range, is necessary to test this hypothesis.

Morphological differences between cytotypes

In the field, different cytotypes of *O. obliquifolia* were visually indistinguishable from one another, but the common garden experiment showed that polyploids are on average larger than diploids. There is some evidence for the gigas effect in polyploids in GCFR *Oxalis* (Becker et al., 2022); however, the size differences are much smaller than most examples of the gigas effect (Stebbins, 1971; Otto and Whitton, 2000; Soltis et al., 2014; Porturas et al., 2019) and are surprisingly inconsistently expressed across *Oxalis* species (Becker et al., 2022). *Oxalis obliquifolia* polyploids showed far more typical and consistent size differences than GCFR congenerics, with effect sizes for size-related characters larger than 20%, consistent with findings by Porturas et al. (2019).

The increased size of *O. obliquifolia* polyploids may have important consequences for physiological processes, such as changes in efficiency of gas exchange, carbon fixation, and water relations (Warner and Edwards, 1993; Levin, 1983, 2002; Vasseur et al., 2022), which can increase plant growth rates, plant vigor, competitive ability, and their ability to respond to abiotic stresses (Van de Peer et al., 2021), but may have trade-offs against other traits. This phenotypic variation is seen as an important aspect in allowing polyploids to potentially adapt and exploit new

TABLE 6 Hurdle model results of seed set as the response variable for the interaction between the type of cross (self-pollinated, intraploid, or interploid) and maternal cytotype (diploid *2x*, tetraploid *4x*, and hexaploidy *6x*), showing the back-transformed estimate, the back-transformed upper and lower 95% confidence intervals (CI), *Z*-, and *P*-values.

	Estimate	Upper 95% CI Lower 95% CI	*Z*	*P*
Zero hurdle model coefficients				
Intercept: Between cytotype:2*x*	0.0588	0.0969 0.03514	−10.064	$<2.0 \times 10^{-16}$*
Self:2*x*	0.0098	0.00138 0.0663	−1.768	7.70×10^{-2}
Within cytotype:2*x*	0.6413	0.7324 0.53867	9.557	$<2.0 \times 10^{-16}$*
Between cytotype:4*x*	0.1625	0.2107 0.12354	3.539	4.02×10^{-4}*
Self:4*x*	0.0354	0.0905 0.01335	0.152	8.79×10^{-1}
Within cytotype:4*x*	0.5780	0.6670 0.48360	−3.233	1.23×10^{-3}*
Between cytotype:6*x*	0.1569	0.2835 0.08044	2.304	2.12×10^{-2}*
Self:6*x*	0.0128	0.0854 0.00180	−0.547	5.85×10^{-1}
Within cytotype:6*x*	0.8000	0.8737 0.69814	−0.483	6.29×10^{-1}
Count hurdle model coefficients				
Intercept: Between cytotype:2*x*	5.81407	8.8658 2.7624	6.580	4.70×10^{-11}*
Self:2*x*	1.08824	3.9822 −1.8057	−1.214	2.25×10^{-1}
Within cytotype:2*x*	19.47950	24.1071 14.8519	4.128	3.67×10^{-5}*
Between cytotype:4*x*	8.46729	10.8788 6.0558	1.240	2.15×10^{-1}
Self:4*x*	3.94611	7.9866 −0.0944	0.615	5.38×10^{-1}
Within cytotype:4*x*	15.60558	19.2284 11.9828	−1.723	8.49×10^{-2}
Between cytotype:6*x*	8.41479	14.0683 2.7613	0.853	3.94×10^{-1}
Self:6*x*	0.00007	0.0174 −0.0173	−0.080	9.37×10^{-1}
Within cytotype:6*x*	13.31569	16.4080 10.2233	−1.612	1.07×10^{-1}

*Significant P-values based on hurdle model results.

ecological niches (Otto and Whitton, 2000). For example, polyploidy may result in larger or differently shaped flowers (Garbutt and Bazzaz, 1983; Balao et al., 2011), which can impact pollinator interactions (Taylor and Smith, 1980; Segraves and Thompson, 1999). It is possible that in *O. obliquifolia*, selection may have preserved the gigas effect for these reasons.

It remains to be seen how variable and, therefore, how useful for cytotype identification, these morphological traits are in situ (phenotypic plasticity; Hahn et al., 2012; Sánchez Vilas and Pannell, 2017). *Oxalis* is notoriously phenotypically plastic (Salter, 1944), and in a wild setting where environmental conditions can vary over a small scale, trait differentiation between different cytotypes may be less pronounced. Alternatively, it may also be the case that competition in the wild may promote differences in morphology.

Phenological shifts

Phenological shifts enable polyploids to escape direct competition with diploids for resources such as light and pollinators (Levin, 2009; Wolkovich and Cleland, 2010). In this study, diploids emerged aboveground slightly earlier and had longer growth periods than polyploids. They also flowered earlier and longer than the tetraploids and hexaploids. Peak flowering times of the tetraploids and hexaploids were concentrated toward the beginning of the diploid flowering season. This phenological patttern is contrary to other reports that flowering of polyploids was distinctly prolonged (Bose and Choudhury, 1962) or delayed (Smith, 1946; Garbutt and Bazzaz, 1983), possibly resulting from slower growth rates associated with the gigas effect. Although the directionality of the shift is unexpected, phenological shifts toward earlier flowering is known to occur in polyploids (Petit et al., 1997; Pyšek et al., 2009; te Beest et al., 2012) and have the potential to facilitate persistence by temporal niche differentiation.

Differences in flowering time are known in systems with sympatric cytotypes (Clark, 1975; Lumaret and Barrientos, 1990; Petit et al., 1997). Although there is evidence

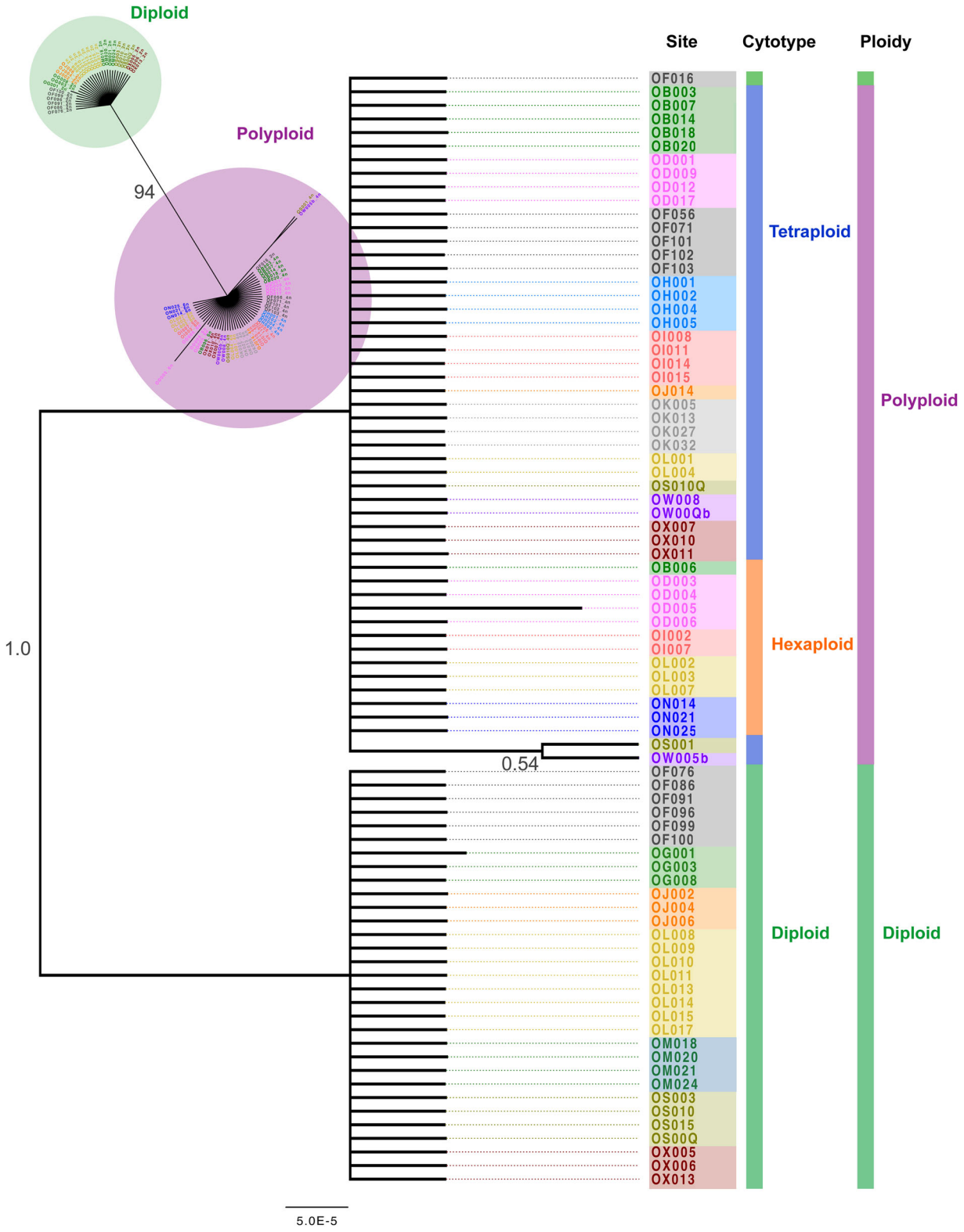

FIGURE 6 Bayesian consensus tree constructed using ITS sequence data, with branch lengths (branch lengths with support under 50 have been collapsed) and posterior probabilities, from *Oxalis obliquifolia* individuals of different cytotypes collected from different sites (same sites indicated using colored tree tips) across Gauteng. Cytotype grouping shown as vertical bars. Inset, top left: the true unrooted tree with branch lengths, with bootstrap support indicated.

TABLE 7 Results of two AMOVA analyses to assess the amount of variation in ribotypes present within, and between, sites and cytotypes as grouping variables. Conducted using ITS sequences of 82 individuals of *Oxalis obliquifolia* (including diploids, tetraploids and hexaploids) and using a distance matrix constructed with the Kimura-2P model.

Source of variation	df	SS	Variance components	Percentage of variation	*P*
AMOVA analysis 1: Grouped by site					
Among sites	13	58.255	−0.10664	−8.12	$<1.0 \times 10^{-4}$*
Among cytotypes, within sites	9	38.310	1.31557	100.21	$<1.0 \times 10^{-4}$*
Within cytotypes	59	6.127	0.10385	7.91	6.39×10^{-1}
Total	81	102.693	1.31279		
AMOVA analysis 2: Grouped by cytotype					
Among cytotypes	2	81.133	1.56464	84.13	$<1.0 \times 10^{-4}$*
Among sites, within cytotypes	20	15.433	0.19131	10.29	$<1.0 \times 10^{-4}$*
Within sites	59	6.127	0.10385	5.58	$<1.0 \times 10^{-4}$*
Total	81	102.693	1.85980		

*Significant P-values.

of an earlier flowering start in polyploids in *O. obliquifolia*, the polyploid flowering period is still completely contained within the longer diploid flowering period. Selection and trait differentiation through pollinator interactions may provide the answer to directional shifts in reproductive traits.

Since polyploidization 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). These impacts can manifest either as a difference in pollinator preference between cytotypes or by cytotypes having different pollinators altogether. An increase in flower size, as 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).

Differences in seed set between cytotypes

Barriers to selfing prevent inbreeding (Heizmann, 1992) and promote genetic diversity in species populations. However, a breakdown in these barriers can facilitate reproductive success for minority cytotypes, thereby mitigating the challenges of MCE. Polyploidization can result in decreased self-incompatibility in outcrossing species (Oswald and Nuismer, 2011; Fowler and Levin, 2016). In this system, polyploidy was not associated with a breakdown of self-incompatibility. This result is consistent with those of previous studies that found self-incompatibility can remain intact in polyploids (see Mable, 2004), contrary to expectations based on studies that have shown otherwise (Husband and Schemske, 1997; Cook and Soltis, 2000).

The associations between cytotype and intraploid seed set can potentially differ dramatically between different species, from higher diploid seed set (Burton and Husband, 2000; Münzbergová and Skuhrovec, 2017) to equivalent seed set across ploidies (Münzbergová, 2007; Castro et al., 2011), to higher polyploid seed set (Černá and Münzbergová, 2013; Gross and Schiestl, 2015). In *O. obliquifolia*, seed set for diploid intraploid crosses was not significantly higher than that of polyploid intraploid crosses. It is notable however that seed set associated with hexaploid intraploid crosses was significantly higher than tetraploid intraploid crosses. This may indicate a fitness advantage for hexaploid individuals that could facilitate establishment among existing tetraploid populations. It remains unknown whether these reproductive dynamics follow the same general pattern under different environmental conditions, or whether trade-offs exist in seed quantity vs quality, which may impact hexaploid fitness.

Interploid crosses were not significantly different from the background selfing rate for diploid and hexaploid maternal cytotypes. However, maternal tetraploid interploid crosses (including both paternal diploids and hexaploids) did have a higher success rate and higher seed set than tetraploid self-pollinations. This would indicate that interploid crosses are possible, but to a lesser extent with maternal diploids, and may potentially yield viable seed (although seed viability and germination did not form part of this investigation; for example in Burton and Husband, 2000). The noticeably low success rate, and seed set associated with interploid crosses with maternal diploids in *O. obliquifolia*, may indicate strong barriers to gene flow from polyploid lineages to diploid lineages.

One major obstacle to successful hybridization 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. This is since gametes produced by triploids are most often non-functional, due to aneuploidy and an imbalance in the number of

chromosomes during meiosis (Satina and Blakeslee, 1937; Dujardin and Hanna, 1988; Hassan and Rehman, 2017). It is notable that no triploid individuals were encountered in this investigation, even at sites where diploids and tetraploids co-occurred. Polyploidization 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 germination success (Ramsey and Schemske, 1998). We could not test seed viability and germination 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, although this would be a fruitful avenue for future research.

Asymmetrical reproductive isolation

As was found in this investigation, other studies have shown that reproductive isolation among higher-ploidy 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, 2017, 2021).

Evidence for this asymmetry was demonstrated in the crossing experiment and was also supported by the ITS population structure, which suggested a clear difference in the degree of gene flow between the polyploid and diploid lineages in natural populations (e.g., Greiner and Oberprieler, 2012). However, ITS markers are subject to the effects of concerted evolution (Alvarez and Wendel, 2003), which ultimately can mask original ITS ribotypes via introgression. Introgression may ultimately overwrite the signal of multiple polyploidization events. Evidence for concerted evolution in our study is quite rare, where only a single minority ribotype was unambiguously observed, which may make multiple polyploidization events, followed by concerted evolution, less likely.

Separate diploid and polyploid lineages suggest the frequency of independent polyploidizations is not rapid enough to explain the high degree of cytotype sympatry. However, the polyploid lineage includes numerous shared ribotypes across higher ploidy cytotypes, suggesting possible hybridization events or independent polyploidization events. 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 notable that the one diploid individual with a polyploid ribotype (accession OF016) was found at the same site as another polyploid individual (accession OF101), which displayed evidence of a second minority ribotype (discernible background sequence) as that of the other diploids. Importantly, these individuals were found at a site where diploids and polyploids co-occur and may potentially indicate a rare instance of in situ gene flow between the polyploid and diploid lineages. However, since this case was the only occurrence of this phenomenon and it is unknown if crosses including these individuals among other diploids would result in viable offspring, it remains to be seen whether evidence will support this potential backward introgression of polyploid genetic material. Furthermore, this 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. These impacts may be due to changes in ecological factors, such as pollinator interactions or abiotic stress, that may result in changes to the frequency of unreduced gamete production and patterns of intercytotype pollination events.

Unidirectional gene flow amongst polyploids has also been found in other polyploid complexes (Greiner and Oberprieler, 2012; Hülber et al., 2015). Strong reproductive isolation between polyploids and their diploid parents may result in differences in diversification rates between the two distinct lineages, particularly when there is homogenizing gene flow among higher-ploidy cytotypes that may result in lower diversification rates (Costa et al., 2014; Sutherland and Galloway, 2017). The presence of reproductive isolation between diploids and polyploid, along with homogenizing gene flow among polyploids, implies that localities with sympatric tetraploids and hexaploids have the potential for increased interploid gene flow when compared with those sites with co-occurring diploids and tetraploids. It is also notable that the only sampled pentaploid individuals were from a mixed tetraploid–hexaploid site. The presence of this pentaploid individual may indicate a "pentaploid bridge" (Peskoller et al., 2021; Šemberová et al., 2021) is involved in the production of higher-ploidy cytotypes and is consistent with our findings of increased seed set in tetraploid–hexaploid crosses.

Furthermore, diploids and polyploids may develop secondary reinforcement to reproductive isolation (Husband and Sabara, 2004) through assortative mating or pollinator-mediated selection (Segraves and Thompson, 1999; Coyne and Orr, 2004; Kennedy et al., 2006). Distinct differences in flower size and phenology between cytotypes, both of which are factors that could allow pollinators to differentiate between cytotypes (Segraves and Thompson, 1999; Husband and Schemske, 2000; Husband and Sabara, 2004) in sympatry, are consistent with this possibility.

CONCLUSIONS

This study offers some unique insights into the patterns of cytogeography and cytotype diversity of a widespread African grassland geophyte and revealed a substantial degree of sympatric cytotype diversity across a relatively small portion of its overall distribution. This high level of sympatry immediately raises the question, how are these different cytotypes able to co-exist so successfully? There is little evidence of niche differentiation across geographic gradients. However, there may be some degree of niche division in time, and there is strong evidence of the gigas effect in this system, with polyploids having larger leaves and flowers in common garden conditions.

Despite the remarkable degree of sympatry in local populations, diploids and polyploids are effectively reproductively isolated from one another in the wild, despite interploid crosses resulting in nontrivial seed set under artificial conditions. It remains unclear how reproductive isolation is maintained in situ. Larger flowers combined with some evidence of slight phenological shifts in flowering time provides intriguing possibilities for pollinator-mediated assortative mating as a potential prezygotic barrier to interploid crosses, although postzygotic effects such as interploid seed fitness also remain untested.

Pollinator interactions and intercytotype competition are two unexplored, yet potentially crucial biotic factors that may facilitate polyploid success and sympatry. Ecological niche shift and direct competition experiments provide promising directions of enquiry for future investigations in this system. Future studies should focus on questions relating to how recently these polyploids have arisen and to what degree vegetative propagation of this species has enabled the persistence of higher-ploidy cytotypes.

This system provides a valuable context for the study of polyploidy as a major contributor to the evolution of angiosperms and has the potential to contribute to existing literature on regional studies regarding the role of polyploidy in intraspecific diversity. This study also highlights the inherent limitations to current taxonomic practices in recognizing real diversity patterns in polyploid complexes, such as in *O. obliquifolia*. One way to address these taxonomic limitations would be to include designations of cytotype as an additional nomenclatural device for the recognition of these intraspecific groups.

AUTHOR CONTRIBUTIONS

All authors contributed to the review and editing of the manuscript. D.V. and K.C.O. conceptualized the project. D.V. wrote the article, gathered data, and prepared figures.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Flow diagram, created by Thien (1979), used in the soil texture characterization of soil samples collected for each individual of *Oxalis obliquifolia* collected in Gauteng Province.

Appendix S2. Color charts used for the categorization of Oxalis obliquifolia floral colors and abaxial leaf surface colors.

Appendix S3. Strong ($|r| \geq 0.7$) trait correlations, indicating traits retained and removed for the univariate analysis of morphological trait variation among cytotypes of *Oxalis 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.

Appendix S4. 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, Akaike information criterion values, and residual deviance values for each parameter combination. Chosen transformations and distribution families are in bold.

Appendix S5. Meiotic chromosome squashes and chromosome counts in pollen mother cells of *Oxalis obliquifolia* and original size and color microscope images of chromosome squashes used to determine chromosome number and morphology.

Appendix S6. Multiple factor analysis plot with colors grouped by cytotype and site and abiotic variable contributions to the construction of the MFA dimensions 1 and 2, showing active (red) and supplementary (green) variables and correlation circle.

Appendix S7. Comparison of the PCA results for the two data sets of morphological characters measured in the common garden experiment, at both the peak of the growing season and 2 months after each plant emerged. Similarities in the identification of important variables in the construction of PC1, PC2 and PC3 are marked in bold (black), and differences have been highlighted in bold and red.

Appendix S8. Summary of data spread and sampling for all crosses performed, indicating the numbers of each type of cross (interploidy, intraploidy, and self-pollinations) and each maternal cytotype (diploid, tetraploid, and hexaploid).

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APPENDIX 1. List of Oxalis obliquifolia voucher specimens for each site included in the study.
Taxon: Plant identifier, Site name (Coordinates), Ploidy, Voucher (PRU accession number).

Oxalis obliquifolia Steud. ex. A.Rich.: OA015, Sable Ranch (S25.93487, E27.61824), diploid, PRU129335. OB001, Miertjie le Roux farm (S25.78102, E28.54816), tetraploid, PRU128128. OC015, Carlswald Estate (S25.97627, E28.10171), tetraploid, PRU129949. OD001, Klipkraal Trails (S26.79635, E28.22789), tetraploid, PRU129942. OE001, Krugersdorp (S26.04579, E27.78981), tetraploid, PRU129943. OF001, Faerie Glen (S25.7742, E28.29369), diploid, PRU128127. OG001, Magaliesburg (S25.80123, E27.99029), diploid, PRU129796. OH003, Hazeldean Trails (S25.77355, E28.40455), tetraploid, PRU129951. OI008, Alberton (S26.30157, E28.07494), tetraploid, PRU129944. OJ001, Fochville (S26.56123, E27.50775), diploid, PRU129945. OK001, Kloofendal Nature Reserve (S26.13077, E27.88219), tetraploid, PRU129952. OL008, Olifantsfontein (S25.94517, E28.17904), diploid, PRU129946. OM001, Moreleta Kloof Nature Reserve (S25.81608, E28.28964), diploid, PRU129950. ON001, Klipreviersberg Nature Reserve (S26.303649, E28.012772), hexaploid, PRU129953. OO001, Cradle Moon Lakeside Lodge (S2595757, E27.86028), tetraploid, PRU129947. OQ009, Windy Brow Game Reserve (S25.68804, E28.50303), tetraploid, PRU129794. OR004, Sandton (S25.8913, E28.23862), tetraploid, PRU128130. OS004, Smuts Koppie (S25.8913, E28.23862), tetraploid, PRU128130. OT001, Muningi Gorge (S25.57704, E28.59107), diploid, PRU129334. OU001, Legends Adventure Farm (S25.82375, E28.55128), diploid, PRU129795. OV001, Kempton Park (S26.06965, E28.26629), tetraploid, PRU129797. OW001, Wonderboom East (S25.69308, E28.20577), tetraploid, PRU128129. OX008, Happy Acres (S26.02610, E27.54644), tetraploid, PRU130792. OY001, Suikerbosrand Nature Reserve (S26.48166, E28.21008), hexaploid, PRU129948.

APPENDIX 2. List of Oxalis obliquifolia GenBank alignment data for ITS DNA sequences of diploid, tetraploid, and hexaploid plants.
Taxon: Site, Ploidy, GenBank accession numbers.

Oxalis obliquifolia Steud. ex. A. Rich.: Miertjie le Roux farm, tetraploid, OP782736, OP782737, OP782738, OP782739, OP782740. Miertjie le Roux farm, hexaploid, OP782773. Klipkraal Trails, tetraploid, OP782741, OP782742, OP782743, OP782744. Klipkraal Trails, hexaploid, OP782774, OP782775, OP782776, OP782777. Faerie Glen, diploid, OP782704, OP782705, OP782706, OP782707, OP782708, OP782709, OP782710. Faerie Glen, tetraploid, OP782745, OP782746, OP782747, OP782748, OP782749. Magaliesburg, diploid, OP782711, OP782712, OP782713. Hazeldean Trails, tetraploid, OP782750, OP782751, OP782752, OP782753. Alberton, tetraploid, OP782754, OP782755, OP782756, OP782757. Alberton, hexaploid, OP782778, OP782779. Fochville, diploid, OP782714, OP782715, OP782716. Fochville, tetraploid, OP782758. Kloofendal Nature Reserve, tetraploid, OP782759, OP782760, OP782761, OP782762. Olifantsfontein, diploid, OP782717, OP782718, OP782719, OP782720, OP782721, OP782722, OP782723, OP782724. Olifantsfontein, tetraploid, OP782763, OP782764. Olifantsfontein, hexaploid, OP782780, OP782781, OP782782. Moreleta Kloof Nature Reserve, diploid, OP782725, OP782726, OP782727, OP782728. Klipreviersberg Nature Reserve, hexaploid, OP782783, OP782784, OP782785. Smuts Koppie, diploid, OP782729, OP782730, OP782731, OP782732. Smuts Koppie, tetraploid, OP782765, OP782766. Wonderboom East, tetraploid, OP782767, OP782768, OP782769. Happy Acres, diploid, OP782733, OP782734, OP782735. Happy Acres, tetraploid, OP782770, OP782771.