

Triploidy causes sexual infertility in *Cyrtanthus breviflorus* (Amaryllidaceae)

Mike Ramsey^{A,C}, Glenda Vaughton^A, Glendon D. Ascough^B and Steven D. Johnson^B

^ABotany, University of New England, Armidale, NSW 2351, Australia.

^BSchool of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville, Pietermaritzburg 3209, South Africa.

^CCorresponding author. Email: mramsey@une.edu.au

Abstract. The balance between sexual and asexual reproduction can vary markedly in clonal plants. At one extreme, plants are sexually infertile and reproduction is solely clonal. Infertility can be caused by environmental and/or genetic factors, but the role of each is often unknown. Here we determine variation in sexual reproduction and explore the underlying factors causing sexual infertility in *Cyrtanthus breviflorus* Harv. We examined open- and cross-pollinated fruit set, ploidy using flow cytometry, pollen viability, pollinator visits to flowers and pollen deposition onto stigmas. One population was sexually infertile; no plants produced fruit. Three populations were sexually fertile; >98% of plants produced fruit. Percent pollen viability differed between infertile (18%) and fertile (97%) populations. The most likely cause of infertility was unequal ploidy. Plants in the infertile population were triploid, whereas those in fertile populations were diploid. Pollination factors were not related to infertility. In infertile and fertile populations, pollen-collecting insects visited flowers frequently, depositing 4-fold more pollen grains onto stigmas than the number of ovules per flower. Our study is the first to demonstrate infertility and triploidy in *C. breviflorus*. How triploidy became established despite high levels of pollinator activity remains a challenging question.

Introduction

Many flowering plant species can reproduce both sexually, by producing seeds that grow into new genetically differentiated plants, and asexually, by producing clonal structures that become independent but genetically identical plants (Richards 1997). Sole reliance on clonal recruitment throughout a population is rare in flowering plants, but can be initiated by either transient or permanent sexual infertility (Dorken and Eckert 2001; Eckert 2002; Honnay and Bossuyt 2005; Silvertown 2008).

Transient sexual infertility is caused by environmental factors when pollinators are absent or unfavourable conditions impede seed production, germination or establishment (Philbrick and Les 1996; Eckert 2002). Genetic factors can also cause transient infertility when *S*-allele or mating-type diversity is reduced in self-incompatible or sexually polymorphic species (Barrett *et al.* 1993; Reinartz and Les 1994; Eckert 2002). Permanent sexual infertility arises most often through changes in chromosome number, particularly uneven ploidy (e.g. triploidy). Triploidy is found in a reasonably large number of plant groups, but spontaneous triploids are usually rare within populations (Grant 1981; Ramsey and Schemske 1998; Husband 2004). Although triploidy can cause sexual sterility (Joly and Bruneau 2004), plants often exhibit low levels of fertility (Ramsey and Schemske 1998; Burton and Husband 2000; Lui *et al.* 2005). The contribution of genetic and environmental genetic factors to sexual infertility is poorly understood. Distinguishing between these factors is necessary to

understand the consequences of and evolutionary pathways to sexual infertility (Barrett *et al.* 1993; Philbrick and Les 1996; Eckert 2002; Dorken *et al.* 2004).

Here we examine causes of sexual infertility in a population of *Cyrtanthus breviflorus* Harv. Our interest in this seemingly typical population was piqued by observations that, unlike other populations, plants appeared not to produce fruit. Previously, we have shown that mating in two sexual populations of *C. breviflorus* is governed by a late-acting self-incompatibility system and that native honeybees (*Apis mellifera scutella*) are major pollinators (Vaughton *et al.* 2010). *Cyrtanthus* Aiton (Amaryllidaceae) is a genus of ~56 species endemic to southern Africa. The genus is characterised by remarkable variation in floral morphology and colour associated with different pollinators, including sunbirds, butterflies, hawkmoths, flies and bees (Snijman and Meerow 2010). Most, if not all, species can reproduce clonally by way of bulbs or bulbils; *C. breviflorus* does not produce bulbils (Reid and Dyer 1984). All 32 species studied to date are diploid ($2n = 2x = 16$), although tetraploid populations have been identified in two taxa, *C. breviflorus* and *C. mackenii* Hook. f. subsp. *mackenii* (both $2n = 4x = 32$; Ising 1969, 1970; Strydom 2005). Of the 23 *C. breviflorus* populations that have been examined 20 were diploid and three were tetraploid. There have been no previous reports of either triploid or sexually infertile populations (Ising 1969; Strydom 2005). However, the presence of diploid and tetraploid populations renders the possibility of

infertile triploid plants originating from diploid \times tetraploid crosses.

To extend our study of reproduction in *C. breviflorus*, we report on variation in sexual fertility among four populations in the KwaZulu-Natal region of South Africa. We determine whether plants in the putatively infertile population are indeed infertile, and if so, whether pollination factors and/or changes in ploidy could be responsible. To address these questions we: (1) quantify infertility by comparing fruit set following natural- and cross-pollination treatments; (2) quantify pollinator visitation to flowers and pollen deposition onto stigmas; (3) compare pollen shape as an indicator of pollen viability; and (4) compare ploidy of plants.

Materials and methods

Study species and sites

Cyrtanthus breviflorus is a geophyte, occurring from the Eastern Cape of South Africa to Kenya. Plants have variable growth forms: a slender form from coastal and inland grasslands and a robust form from inland marshes. Phenotypic differences between forms disappear under common garden conditions. Flowering is after early spring fires. Inflorescences are umbellate with two to eight yellow campanulate flowers. Flowers are erect with styles that extend beyond the anthers. Fruit are capsules, with flat, winged seeds (Reid and Dyer 1984).

Our main interest was a population of putatively infertile plants of the robust form growing beside a wetland adjacent to exotic pine plantations near Mt Gilboa in the Karkloof Range (29°16.462'S, 30°17.070'E; 1620 m above sea level). The population had at least 2000 plants in an area \sim 10 by 100 m. We also examined three populations of fertile plants: two of the robust form from the Drakensburg Range (Giants Castle: 29°16.054'S, 29°41.255'E; 1580 m above sea level; Highmoor: 29°19.325'S, 29°37.719'E; 1940 m above sea level), and one of the slender form from Pietermaritzburg (Campus: 29°37.713'S, 30°24.020'E; 700 m above sea level). Giants Castle and Campus populations contained 700–1000 plants, but the Highmoor population was much smaller ($n=17$ plants). We conducted the study during September–November 2009. All plants had one to four scapes, each with two to six flowers. Flowers typically opened in the morning, closed in the evening and reopened each morning for 2 or 3 days.

Natural and cross fruit set

We assessed whether plants produced fruit under natural conditions in all populations. In the Mt Gilboa and Campus populations, we marked with flagging tape 250 and 76 plants, respectively, during flowering and later assessed whether at least one fruit was produced. At Giants Castle, we walked along haphazardly selected transects and at 2-m intervals assessed whether the nearest plant had produced at least one fruit ($n=112$ plants). We assessed all plants in the Highmoor population ($n=17$). We assessed fruit set per plant as fruit/flowers per scape on 50 plants at Mt Gilboa, Campus and Giants Castle and all plants at Highmoor.

In the Mt Gilboa and Campus populations, we manually cross-pollinated flowers to establish whether plants could produce fruit. At Mt Gilboa, we assigned plants to two cross-

pollinated treatments. (1) Cross-pollination: as flowers opened, they were emasculated and cross-pollinated, and scapes were bagged with fine mesh for \sim 48 h to exclude pollinators until flowers wilted ($n=25$ plants). (2) Supplementary cross-pollination: as flowers opened, they were cross-pollinated, but not emasculated or bagged, allowing natural pollination ($n=40$). In the Campus population, we conducted cross-pollination treatment (1) only ($n=35$). In both populations, we treated only the first flower on plants, removing other flowers after they wilted to reduce the likelihood of resources limiting fruit set. Each flower was pollinated using a different combination of two donors at least 5 m distant. At Mt Gilboa and Campus 130 and 70 pollen donors were used, respectively. Five weeks later we assessed fruit set.

Ploidy analyses

We estimated DNA ploidy level of plants from the Mt Gilboa ($n=8$), Giants Castle ($n=5$) and Campus ($n=5$) populations using flow cytometry. Plants were excavated from the field, transferred to the laboratory and stored at 5°C. Within 24 h, we obtained nuclear suspensions from fresh young leaves of each plant following the protocols of Dolezel and Bartos (2005). Nuclei were released by chopping 1–2 cm² of leaf tissues with a razor blade in a Petri dish containing 0.5 mL of Otto I solution [100 mM citric acid, 0.5% (v/v) Tween 20]. We filtered the nuclear suspension using an 80- μ m nylon filter into a cytometer sample tube and added 1 mL of Otto II solution (400 mM Na₂PO₄.12H₂O) for staining at neutral pH. For staining, we used 50 mg/mL of fluorochrome propidium iodide. Samples were kept on ice and analysed within 45-min in a Coulter EPICS XL-MCL (Coulter Electronics, Hialeah, FL, USA) flow cytometer equipped with an air-cooled argon-ion blue laser tuned at 15 mW and operating at 488 nm. We removed doublets from data analysis using a region defined in a fluorescence light pulse integral versus fluorescence light pulse height cytogram that included only the particles of interest. For each sample, we calculated peak means and coefficients of variation from between 1500 and 3000 nuclei (i.e. fluorescent events).

Pollen sterility

In the Mt Gilboa, Giants Castle and Campus populations, we removed two anthers from a flower on each of 10 plants and dabbed each onto individual cubes of glycerin gel with basic fuchsin on microscope slides. We added coverslips, melted and squashed the gels and counted at least 200 pollen grains per slide at 40 \times magnification. We did not test viability *per se*, but assumed that misshapen grains were non-viable. We compared percent pollen viability using a one-way ANOVA, following arcsine square-root transformation.

Do pollinators and pollen deposition limit fruit set?

We observed insects visiting single flowers on different plants for three 30-min periods between 0900 and 1300 hours on each of 2 days at Mt Gilboa and for one 30-min period between 0900 and 1200 hours on each of 6 days at Campus. All flowers had opened on the day of observation and different flowers were observed on each day ($n=20$ and 18 flowers at Mt Gilboa and

Campus, respectively). We recorded the number of individuals belonging to three functional groups (honeybees, syrphid flies, solitary bees) and calculated the number of visits/h by each group to each flower. We conducted observations on sunny days; insects did not visit flowers on rainy days. For 5 days at Mt Gilboa and 8 days at Campus, we assessed whether floral visitors contacted stigmas by opportunistically observing flowers between 0900 and 1400 hours. We also noted whether visitors collected pollen.

In the Mt Gilboa and Campus populations, we counted the number of pollen grains deposited on naturally pollinated stigmas ($n=14$ and 12 flowers, respectively). Large anther-stigma distances prevent autonomous self-pollination, and only insects deposit pollen. We marked flowers as they opened, left flowers untouched for 24 h and then collected pistils. We placed excised stigmas onto separate cubes of glycerin gel with basic fuchsin on a microscope slide, added a coverslip and melted and squashed the gel. We counted the number of pollen grains on each stigma at $40\times$ magnification. We compared pollen deposition from the two populations using a one-way ANOVA. We also counted the number of ovules/flower, and calculated pollen grain : ovule ratios.

Results

Natural and cross fruit set

In the Mt Gilboa population, neither open- nor cross-pollinated plants produced fruit. By contrast, in the other populations more than 98% of open-pollinated plants produced fruit and fruit set per scape was $\sim 60\%$ (Table 1). In the Campus population, 94.7% of cross-pollinated flowers produced fruit, and the seed : ovule ratio was 0.72 ± 0.03 .

Ploidy analyses

Flow cytometric analysis of DNA ploidy provided histograms with well defined peaks. On average, plants from Mt Gilboa had peak values ~ 1.45 and 1.42 times greater than peaks of plants from Giants Castle and Campus, respectively. These peaks indicate that plants from Mt Gilboa were triploid, whereas plants from Giants Castle and Campus were diploid (Fig. 2). Although peaks were well defined, small differences in sample quality were detected. Coefficients of variation ranged from 2.6 to 6.9% for Mt Gilboa plants, 1.3 to 7.7% for Giants Castle plants and 3.7 to 7.2% for Campus plants. Some of these coefficients of variation are marginally greater than the 5% suggested by Dolezel and Bartos (2005).

Pollen sterility

In the Mt Gilboa population, most pollen was misshapen and probably sterile (Fig. 1). Pollen viability of Mt Gilboa plants was only $\sim 20\%$ that of Giants Castle and Campus plants ($F_{2,27}=250.90$, $P<0.0001$, Table 1).

Do pollinators and pollen deposition limit fruit set?

In the Mt Gilboa population, 46.7% of floral visitors were syrphid flies, 30.0% were honeybees and 23.3% were solitary bees ($n=137$). The mean number of visits/flower per h (\pm s.e.) by the three groups was: syrphid flies, 2.1 ± 0.3 ; honeybees, 1.4 ± 0.3 ; solitary bees, 1.1 ± 0.2 . In the Campus population, we observed no syrphid flies and 91.7% of floral visitors were honeybees and 8.3% were solitary bees ($n=146$). These two groups made 12.7 ± 1.3 and 1.1 ± 0.4 visits/flower per h, respectively.

We assessed whether floral visitors contacted stigmas by observing 147 honeybees, 134 syrphid flies and 115 solitary bees at Mt Gilboa and 141 honeybees and 24 solitary bees at Campus. Most syrphid flies (71%) and honeybees (Mt Gilboa, 81%; Campus, 92%) contacted stigmas during foraging. Only 10% (Mt Gilboa) and 13% (Campus) of solitary bees did so, owing to their smaller sizes. All visitors at Mt Gilboa and all honeybees and 92% of solitary bees at Campus collected pollen from flowers. Pollen deposition onto stigmas did not differ significantly between the Mt Gilboa and Campus populations ($F_{1,24}=0.642$, $P=0.221$, Table 1). Pollen deposition exceeded ovule number by 3.7- and 4.5-fold, respectively.

Discussion

Our study is the first to document sexual infertility and triploidy in *C. breviflorus*. We verified that plants in the Mt Gilboa population were triploid, produced mostly non-viable pollen, and failed to produce fruit when flowers were pollinated naturally or manually with cross pollen. By contrast, plants in the other populations were diploid and sexually fertile. In these populations, more than 98% of plants produced fruit and fruit set of individual plants was $\sim 60\%$. These findings indicate that in the Mt Gilboa population recruitment must be by asexual propagation (i.e. cloning), whereas in the sexual populations both seeds and cloning can contribute to recruitment. Whether the Mt Gilboa population is composed of a single triploid clone or multiple clones remains to be determined.

Lack of pollination in the asexual Mt Gilboa population was unlikely to have caused sexual infertility. Despite large

Table 1. Fruit set, pollen viability, pollen grains per stigma, and ovules per flower, for the sexually infertile (Mt Gilboa) and fertile (Giants Castle, Highmoor and Campus) populations of *Cyrtanthus breviflorus*

The number of plants assessed is given in parentheses. Except for the percentage of plants with fruit, means \pm s.e. are given. Not all traits were examined in all populations

	Mt Gilboa	Giants Castle	Highmoor	Campus
Plants with fruit (%)	0.0 (200)	99.1 (112)	100.0 (17)	98.7 (76)
Fruit set/scape (%)	0.0 ± 0.0 (200)	59.4 ± 3.1 (50)	64.0 ± 4.7 (17)	58.3 ± 3.2 (50)
Pollen viability (%)	17.8 ± 3.9 (10)	96.9 ± 0.7 (10)	–	97.2 ± 0.5 (10)
Pollen grains/stigma	207 ± 26 (14)	–	–	186 ± 18 (12)
Ovules/flower	56.0 ± 2.7 (15)	–	–	40.9 ± 1.7 (15)

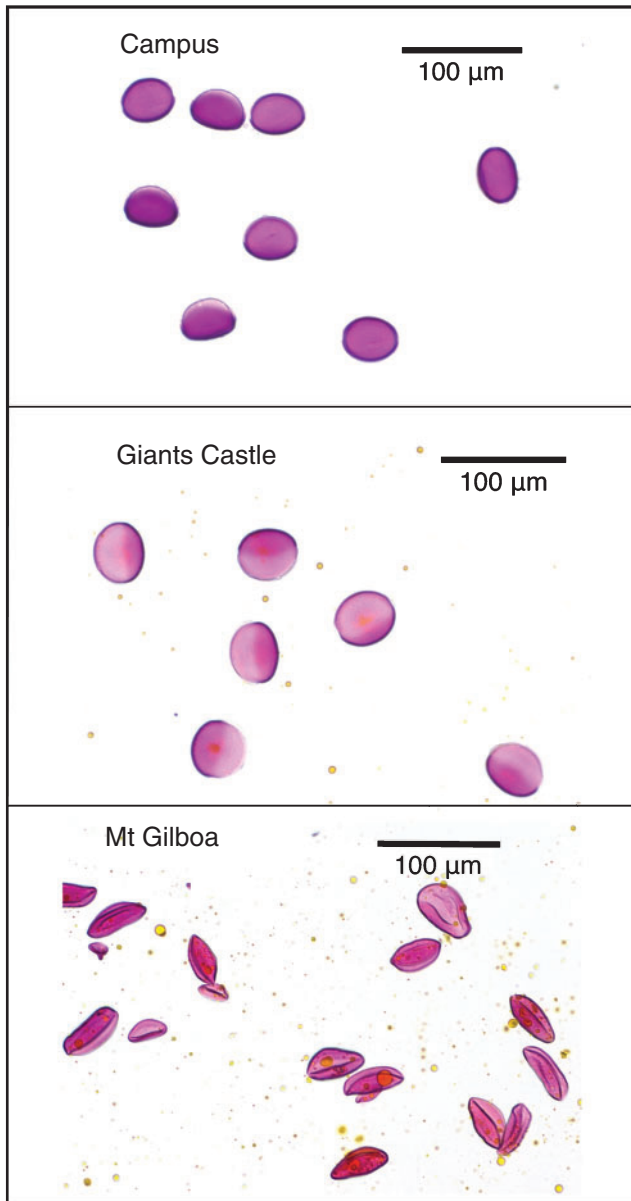


Fig. 1. Pollen grains from the sexually fertile Campus and Giants Castle populations and the infertile Mt Gilboa population of *Cyrtanthus breviflorus*. Misshapen grains were considered non-viable.

numbers of visits by honeybees and syrphid flies to flowers and substantial pollen deposition onto stigmas, none of the open-pollinated plants that we marked produced fruit (total $n = 250$ plants). Further, on several occasions we walked extensively through the population assessing plants ($n > 500$), but found no fruit. Finally, none of the experimentally cross-pollinated plants produced fruit, despite testing 130 plants as pollen donors and 65 plants as pollen recipients. This contrasts with the sexual populations, in which almost 99% of open-pollinated plants produced fruit. Overall these findings demonstrate that sexual infertility in the Mt Gilboa population is unlikely to be related to limitation by pollen quantity. However, we did not examine directly whether pollen quality caused sexual infertility, as

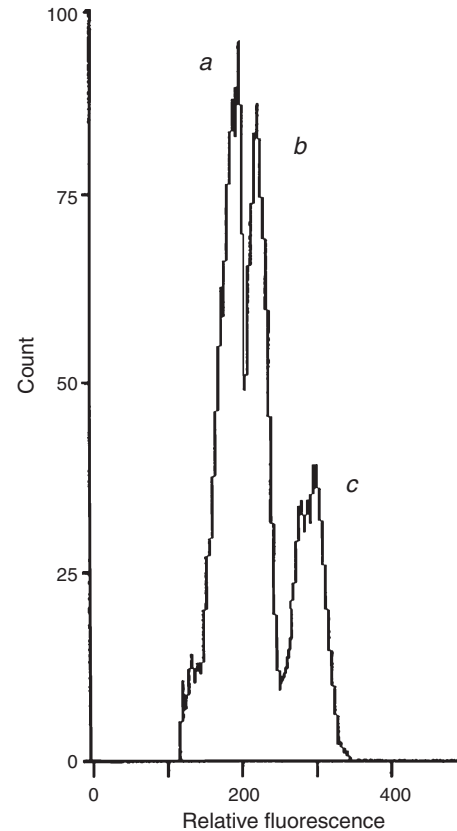


Fig. 2. Ploidy estimates from flow cytometry in *Cyrtanthus breviflorus*. Relative fluorescence intensity obtained after analysis of nuclei isolated from *C. breviflorus*. Plants were from Giants Castle (a), Campus (b) and Mt Gilboa (c) populations. The mean relative fluorescence and coefficient of variation (%) representing the population peaks were: Giants Castle, 191, 5.7%; Campus, 208, 4.2%; and Mt Gilboa, 292, 5.4%. Differences between peaks indicate that plants from Giants Castle and Campus are diploid and plants from Mt Gilboa are triploid.

may be the case if the population is dominated by one or a few clones (Honnay *et al.* 2006). In the Campus population, plants exhibit a late-acting self-incompatibility mechanism, and high levels of self-pollination can cause pollen limitation of seed set (Vaughton *et al.* 2010). Low mating type (*S*-allele) diversity in the Mt Gilboa population could potentially contribute to the observed sexual infertility. Reciprocal crossing experiments between plants from Mt Gilboa and other broad-leaved populations of *C. breviflorus* could be undertaken to determine whether plants from Mt Gilboa are capable of producing seeds and whether pollen of plants from Mt Gilboa is partially fertile, as our findings indicate.

The most likely cause of infertility in the Mt Gilboa population was a change from diploidy to triploidy. We assessed ploidy using flow cytometry and found that relative DNA content of plants from infertile Mt Gilboa was ~ 1.5 -fold greater than that of plants from the fertile Campus and Giants Castle populations. Based upon cytology work by Ising (1969, 1970), plants from the Campus and Giants Castle populations are diploid ($2n = 2x = 16$), and plants from the Mt Gilboa population are triploid ($2n = 3x = 24$) or near-triploid aneuploids (e.g. $3x \pm 1-3$). Our

findings raise the question of the origin of triploidy in the Mt Gilboa population. There are two major pathways to triploidy: (1) crosses between diploid plants that produce both unreduced ($2n$) gametes and reduced (n) gametes; and (2) crosses between diploid ($2n$) and tetraploid plants ($4n$). The union of reduced and unreduced gametes is probably the most frequent pathway to triploidy (Grant 1981; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). However, crosses between diploid and tetraploid plants as an explanation for triploidy in the Mt Gilboa population may be more likely.

Although chromosome number is normally stable in *Cyrtanthus* ($2n=2x=16$; Ising 1970), tetraploidy has been found in three populations of *C. breviflorus* (all $2n=4x=32$; Ising 1969). Based on chromosome morphology, Ising (1969) speculated that these plants were autotetraploids, resulting from diploid crosses between differently adapted ecotypes. These tetraploid populations are found in districts located within 100–200 km of the Mt Gilboa population in north, north-east and south-east directions, and in at least one of these districts, both diploid and tetraploid plants have been found (see table 1 in Ising 1969). Further, although we are the first to document triploidy in a natural population of *C. breviflorus*, Ising (1969) experimentally produced triploids and near-triploid aneuploids by crossing diploid and tetraploid plants in a glasshouse environment. Seeds from these crosses germinated, but sexual fertility of the resulting plants was not assessed. We suggest that the reasonably close proximity of natural diploid and tetraploid populations increase the probability of diploid \times tetraploid crosses and the subsequent formation of triploids under natural conditions. Further, occasional pollen flow between different diploid and tetraploid plants could result in multiple triploid clones existing in this population [c.f., *Lomatia tasmanica* (Proteaceae), Lynch *et al.* 1998].

Estimated pollen fertility of plants in the Mt Gilboa population was substantially less than that of the sexual populations (18 v. 97%; Table 1). Pollen fertility is typically low in triploids and is caused by the production of aneuploid gametes with unbalanced chromosome numbers, owing to problems with chromosomal pairing and segregation during meiosis. Variability in gamete cytotypes is considerably greater for uneven ploidy cytotypes (e.g. $3n$, $5n$) than for even ploidy cytotypes (e.g. $2n$, $4n$). Nevertheless, low levels of pollen fertility, such as we found in the Mt Gilboa population, could be expected because triploids often generate small numbers of euploid (x , $2x$) gametes as well as triploid gametes via non-reduction (Ramsey and Schemske 1998). If some pollen of plants from Mt Gilboa is indeed fertile and if meiotic segregation is similar for male and female gametes, then triploids could be expected to produce some fertile diploid and/or tetraploid progeny through the union of euploid gametes or euploid and unreduced gametes. The probability of such events, however, is quite low (Ramsey and Schemske 1998, 2002; Otto and Whitton 2000), and could explain the lack of sexual fertility in the Mt Gilboa population. On the other hand, fertile or partially fertile individuals might be present, but low mating type diversity renders all crosses ineffective. This would imply that a population bottleneck occurred either in association with or shortly after the introduction of triploidy.

Several scenarios are plausible to explain the occurrence of triploidy in the Mt Gilboa population. Genome increase could

have increased ecological tolerance and/or survivorship of triploids compared with fertile diploid plants. Similarly, because triploids do not produce fruit or seeds, any resource reallocation from sexual functions that enhanced clonal growth or ramet survival may have provided triploids with a competitive advantage. For example, in *Chamerion angustifolium* (Onagraceae), Burton and Husband (2000) produced triploid progeny by crossing diploids and tetraploids. They found that although triploid fertility was less than that of diploids, plant growth was greater. In *C. breviflorus* at Mt Gilboa, such advantages, even if small, could have been important in increasing triploid frequency during the establishment of the surrounding exotic pine plantations. The accompanying habitat disturbance could have caused reductions in population size that reduced the probability of sexual reproduction, favouring clonality.

Overall our findings demonstrate that sexual infertility is most likely caused by triploidy. Pollination factors are unlikely to contribute to this infertility, and in fact, provide sufficient pollination consistent with high levels of sexual reproduction in other populations. Several questions remain unanswered by our study. One of the questions is the number of genotypes (genotypic diversity) in the infertile Mt Gilboa population. Is this population composed of a single clonal genotype or multiple clonal genotypes? Future studies that incorporate molecular markers would be most informative in addressing this question (e.g. Eckert *et al.* 2003). Given the apparent absence of sexual plants from the Mt Gilboa population, studies that compare ecological tolerance, plant growth and rate of clonal reproduction in sexually fertile diploid and infertile triploid forms would also be valuable. Finally, a much broader sample of populations is required to investigate the extent that sexual fertility varies among populations of *C. breviflorus*.

Acknowledgements

We thank S-L. Steenhuisen for much assistance with the project and the EM unit staff at UKZN for assistance with microscopy.

References

- Barrett SCH, Eckert CG, Husband BC (1993) Evolutionary processes in aquatic plants. *Aquatic Botany* **44**, 105–145. doi:10.1016/0304-3770(93)90068-8
- Bretagnolle F, Thompson JD (1995) Tansley Review no. 78. Gametes with the somatic chromosome number: the mechanisms of their formation and the role in the evolution of autopolyploid plants. *New Phytologist* **129**, 1–22. doi:10.1111/j.1469-8137.1995.tb03005.x
- Burton TL, Husband BC (2000) Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustiflorum*: mechanisms of inviability and implications for polyploidy evolution. *Evolution* **54**, 1182–1191.
- Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany* **95**, 99–110. doi:10.1093/aob/mci005
- Dorken ME, Eckert CG (2001) Severely reduced reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). *Journal of Ecology* **89**, 339–350. doi:10.1046/j.1365-2745.2001.00558.x
- Dorken ME, Neville KJ, Eckert CG (2004) Evolutionary vestigialization of sex in a clonal plant: selection versus neutral mutation in geographically peripheral populations. *Proceedings. Biological Sciences* **271**, 2375–2380. doi:10.1098/rspb.2004.2875

- Eckert CG (2002) The loss of sex in clonal plants. *Evolutionary Ecology* **15**, 501–520. doi:10.1023/A:1016005519651
- Eckert CG, Lui K, Bronson K, Corradini P, Bruneau A (2003) Population genetic consequences of extreme variation in sexual and clonal reproduction in an aquatic plant. *Molecular Ecology* **12**, 331–344. doi:10.1046/j.1365-294X.2003.01737.x
- Grant V (1981) 'Plant speciation.' 2nd edn. (Columbia University Press: New York)
- Honnay O, Bossuyt B (2005) Prolonged clonal growth: escape route or route to extinction? *Oikos* **108**, 427–432. doi:10.1111/j.0030-1299.2005.13569.x
- Honnay O, Jacquemyn H, Roldán-Ruiz I, Hermy M (2006) Consequences of prolonged clonal growth on local and regional genetic structure and fruiting success of the forest perennial *Maianthemum bifolium*. *Oikos* **112**, 21–30. doi:10.1111/j.0030-1299.2006.14077.x
- Husband BC (2004) The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. *Biological Journal of the Linnean Society. Linnean Society of London* **82**, 537–546. doi:10.1111/j.1095-8312.2004.00339.x
- Ising G (1969) Cytogenetic studies in *Cyrtanthus*. IV. Chromosome morphology in *Cyrtanthus luteus* Baker (*Anoiganthus luteus* Baker) and *Cyrtanthus breviflorus* Harv. (*Anoiganthus breviflorus* Baker). *Hereditas* **63**, 352–384. doi:10.1111/j.1601-5223.1969.tb02267.x
- Ising G (1970) Evolution of karyotypes in *Cyrtanthus*. *Hereditas* **65**, 1–28. doi:10.1111/j.1601-5223.1970.tb02305.x
- Joly S, Bruneau A (2004) Evolution of triploidy in *Apios americana* (Leguminosae) revealed by genealogical analysis of the histone H3-D gene. *Evolution* **58**, 284–295.
- Lui K, Thompson FL, Eckert CG (2005) Causes and consequences of extreme variation in reproductive strategy among invasive populations of a clonal aquatic plant, *Butomus umbellatus* (Butomaceae). *Biological Invasions* **7**, 427–444. doi:10.1007/s10530-004-4063-3
- Lynch AJJ, Barnes RW, Cambecèdes J, Vaillancourt RE (1998) Genetic evidence that *Lomatia tasmanica* (Proteaceae) is an ancient clone. *Australian Journal of Botany* **46**, 25–33. doi:10.1071/BT96120
- Otto SP, Whitton J (2000) Polyploid incidence and evolution. *Annual Review of Genetics* **34**, 401–437. doi:10.1146/annurev.genet.34.1.401
- Philbrick CT, Les DH (1996) Evolution of aquatic angiosperm reproductive systems. *Bioscience* **46**, 813–826. doi:10.2307/1312967
- Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploidy formation in flowering plants. *Annual Review of Ecology and Systematics* **29**, 467–501. doi:10.1146/annurev.ecolsys.29.1.467
- Ramsey J, Schemske DW (2002) Neoploidy in flowering plants. *Annual Review of Ecology and Systematics* **33**, 589–639. doi:10.1146/annurev.ecolsys.33.010802.150437
- Reid C, Dyer RA (1984) 'A review of the southern African species of *Cyrtanthus*.' (American Plant Life Society: La Jolla, CA)
- Reinartz JA, Les DH (1994) Bottleneck-induced dissolution of self-incompatibility and breeding system evolution in *Aster furcatus* (Asteraceae). *American Journal of Botany* **81**, 446–455. doi:10.2307/2445494
- Richards AJ (1997) 'Plant breeding systems.' 2nd edn. (Chapman and Hall: London)
- Silvertown J (2008) The evolutionary maintenance of sexual reproduction: evidence from the ecological distribution of asexual reproduction in clonal plants. *International Journal of Plant Sciences* **169**, 157–168. doi:10.1086/523357
- Snijman DA, Meerow AW (2010) Floral and macroecological evolution within *Cyrtanthus* (Amaryllidaceae): inferences from combined analyses of plastid *ndhF* and nrDNA ITS sequences. *South African Journal of Botany* **76**, 217–238. doi:10.1016/j.sajb.2009.10.010
- Strydom A (2005) Phylogenetic relationships in the family Amaryllidaceae. PhD Thesis, University of the Free State, Bloemfontein, South Africa.
- Vaughton G, Ramsey M, Johnson SD (2010) Pollination and late-acting self-incompatibility in *Cyrtanthus breviflorus* (Amaryllidaceae): implications for seed production. *Annals of Botany* **106**, 547–555. doi:10.1093/aob/mcq149

Manuscript received 14 October 2010, accepted 28 February 2011