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Population structure, genetic diversity and conservation of selected species of Barleria

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Red Data assessment, allozyme diversity, reproductive biology and management strategies in populations of a rare and restricted species, *Barleria greenii* (Acanthaceae).

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Abstract

Barleria greenii is a rare narrow endemic confined to Estcourt (South Africa) with populations occurring in areas under three different management regimes: a two year burning cycle with moderate grazing, a four year burning cycle with very light grazing and an annual burning cycle with low grazing. Its population dynamics including population genetic structure using allozyme markers, IUCN Red Data Assessment, type of breeding system and reproductive effort, were studied. The type of management strategy that best suits *B. greenii* was determined. Low within and among population variation and differentiation, mixed mating systems and an IUCN conservation rating of Vulnerable (VU D2) were found. The two year burning cycle with moderate grazing was found to be the most appropriate management regime for *B. greenii* with overall density and density of juveniles and seedlings exceeding that in the four year burning cycle. Canopy area, volume index and biomass including grass, forbs and litter but excluding *B. greenii*, were low in the two year burning cycle. In addition there were some individuals in which the above ground parts (stems) survived fire in the two year burning cycle as a result of cooler fires due to high stocking rates and hence less biomass of grass, forbs and litter. The conservation rating, allozyme variation and differentiation, breeding systems and management regimes indicated that the species is threatened and should be of major concern.

Introduction

Barleria greenii is a South African perennial shrub that grows exclusively in doleritic soils. The species has a restricted distribution at Estcourt (Figure 1) and is listed as vulnerable in the Red Data Book (Hilton-Taylor, 1996) but the area of occupancy and the extent of occurrence were unknown when the status of this plant was determined. Total number of populations and individuals might also have been underestimated. The sites where *B. greenii* occurs are well protected at present, falling either within the boundaries of the Lowlands East Conservancy or within the Weenen Nature Reserve. The areas in which the populations in the Lowlands East Conservancy occur are privately owned, grazed by cattle and burned every two years whereas the areas in the Weenen Nature Reserve are very lightly grazed and burned every four years. The management strategy regime best suits *B. greenii* is unknown.

Balkwill et al. (1990) described flower phenology in *B. greenii*. In general, flowers of *B. greenii* are very conspicuous, protogynous, nocturnally scented and flower from January to May with colour ranging from white to deep pink. The corolla is five lobed and the sticky stigma is held above the anthers. The fruit is a four-seeded capsule. Hawkmoths have been predicted as potential pollinators (Balkwill et al., 1990). Nothing further is known about floral biology and pollination of the species in this genus but a considerable amount of work has been done in the family Acanthaceae in the genera *Eranthemum* (Scott, 1872), *Justicia* (McDade and Kinsman, 1980), *Ruellia* (Lord, 1981), *Aphelandra* (McDade, 1985; Calvo-Irabien and Islas-Luna, 1999), *Pseuderanthemum* (Balkwill and Balkwill 1999), *Thunbergia* (Schonenberger, 1999), *Dendrobium* (Ketsa *et al.,* 2001) and neotropical Acanthaceae (Vogel *et al.,* 2004).

The species also displayed low levels of allozyme diversity (Makholela et al., 2003) in a preliminary investigation of the amounts of genetic variability on three populations. Therefore, the aim of this study was to re-assess the Red Data Status of *B. greenii*, assess which management strategy was most appropriate for *B. greenii*, determine its breeding system, reproductive effort and population structure including both the population genetic structure of other known populations and the demographic structure in order to provide advice on management of this species that would favour its conservation.

Materials and methods

Red data assessment (IUCN Categories) and management regimes

Occurrences of plants were defined as distinct populations if they were separated by discontinuities of at least 1km (Keith, 1998) as a result of short distance seed dispersal in these plants. Groups of individuals separated by less than 1km were regarded as subpopulations. This level of geographical isolation seems appropriate for plant species whose propagules are dispersed over metres (Morgan 1995) or tens of metres (Lamont 1985; Hammill et al., 1998). In *B. greenii*, there is short distance seed dispersal (less than 5m) (K. Balkwill, pers. comm.) with no structures to assist secondary dispersal by animals, wind or water. Every existing collection is either cited in the paper by Balkwill et al. (1990) or the duplicate is available at C. E. Moss Herbarium (J). Flowering periods were compiled from dates on herbarium labels. Data were collected from all known populations.

Plants from all known populations were counted and categorized as adults (all reproductive plants as well as those that were not reproductive but larger than the smallest reproductive plant), juveniles (all plants smaller than the smallest reproductive plant) and seedlings (all plants with one above ground shoot). GPS recordings of the outermost individuals were made while walking along the edges of each population. This information was used to calculate the area of occupancy and the

extant of occurrence using IDRISI (Eastman 1997), a Geographical Information System program. The RAMAS Version 2.0 program (Akçakaya and Ferson 2001), which implement IUCN threatened species criteria, was used to determine the IUCN Red Data Status.

Population numbering followed Balkwill et al. 1990 except for Populations which have either been united to form one population or have been reduced to subpopulations. For these exceptional populations or subpopulations new population numbering is proposed and Balkwill et al. 1990 populations numbering is given in parenthesis. For instance a number without parenthesis denotes that the population numbering in the present study and the study by Balkwill et al. 1990 is the same, whereas 2a (8a and 8b) shows that Population 8a and 8b from Balkwill et al. 1990 have been united to form one population (2a). On the other hand 4a(4) means that population 4 in Balkwill et al. 1990 numbering system have been reduced to Subpopulation 4a in the present study.

Barleria greenii populations occur in areas with different fire and grazing regimes. Populations 1, 2a, 3, 4a, 4b, 4c, 9 are moderately grazed, burned every two years and occur on privately owned land. Populations 2 and 6 are found in the Weenen Nature Reserve and are lightly grazed and burned every four years (Figure 1). Population 7 is composed of three different burning regimes. Part of Population 7 is an annual burn (fire break). The other part of Population 7 is burned every two years and there is an area burned every four years within the same population. Both the firebreak and the four year burning cycle are found in the Weenen Nature Reserve. These are separated by a game fence from a two year burning cycle which extends outside the Nature Reserve into the privately owned land. Livestock grazes on the privately owned land but there are only very low stocking rates in the Nature Reserve.

Management strategy, in terms of fire regime and grazing level, most appropriate for *B. greenii* was determined from all known populations in the Weenen Nature Reserve (Populations 2, 6 and part of Population 7 consisting of the firebreak and the four year burning cycle). Two Populations (2a and 3) and part of Population 7 in the privately owned land were used to determine the most appropriate management strategy for populations in the two year burning cycle. In addition to different burning regimes for the above mentioned populations, the time since fire was different regardless of whether the populations were in the same or different burning regimes. Population 6 and part of Population 7 in the Weenen Nature Reserve were four years post fire whereas Populations 2 (also from the Weenen Nature Reserve) was two years post fire in the four year burning cycle. For populations in the two year burning cycle Population 3 was a two years post fire, Populations 2a and part of Population 7 were one year post fire.

To determine the most appropriate strategy for *B. greenii* 15m by 15m (total of eight $225m^2$ quadrats for each treatment and 56 quadrats for all treatments) were used to study density of seedlings, juveniles, adults and overall density by counting each plant from each quadrat. The canopy area, volume index and height were also measured for each plant in each quadrat whereas reproductive effort was determined from one medium plant from each quadrant. Plant size was quantified as canopy area calculated according to Witkowski *et al.* (1994) from the widest diameter of each plant (W_1) in a quadrat and the diameter perpendicular to it (W_2) where canopy area = $\prod (W_1/2)(W_2/2)$. Canopy area was then multiplied by height to get volume.

For reproductive effort, plants were placed in a bag and brought to the laboratory in order to count the number of leaves including the ones in which the leaves had fallen off, calyces and fruit set. Leaves, calyces and fruits were dried and weighed in order to determine vegetative and reproductive mass which were used to calculate Reproductive Effort as: Reproductive mass/Total mass x 100%. In addition, biomass of grass, forbs and litter but excluding *B. greenii* was estimated by cutting the vegetation in ten $1m^2$ quadrats located randomly within the boundaries of each population, followed by drying and measuring the dry weight. Canopy area and volume index were represented as histograms. Correlation between canopy area and height was determined in different burning regimes.

Reproductive biology, fruit set and nectar sugar composition

Observations on how the insects interact with flowers were made during the day and at night in order to determine the pollinating agents of *B. greenii*. It was noted whether flower visitors made contact with the stigmas or the anthers and the way in which they access the resources in the flower. Insects were killed in a killing bottle charged with ethyl acetate, pinned and mounted for identification.

Visitor observations were made in Populations 8(2a), 3, 4(4a) and 5(4c) (Figure 1) in February 2003. A total of approximately 12 hours was spent during these observations. The proboscides of the Lepidopteran (n = 8) visitors and the carpenter bee (n = 1) were uncoiled and measured. Insects from the field were viewed under a Nikon SMZ 1500 light microscope connected to a Nikon Digital Camera DXM 2000 for presence/absence of pollen grains. Insect identification was corroborated by sending specimens to a specialist (Dr Martin Kruger, Transvaal Museum).

In addition, Pollen:Ovule ratio (P:O) (Cruden 1977) was determined in five young flower buds by cutting the anthers into small pieces. Each of these pieces was viewed under a microscope on a slide and 70% ethanol was added to allow pollen grains to be spaced throughout the slide. Cruden's (1977) outcrossing index was also used to determine the type of breeding system. Ovule (big enough to be seen with a naked eye) count was made from fruits.

Field estimates of nectar volume were made from flowers bagged with shade net growing in natural populations at Estcourt (South Africa) during January to February 2003. Flowers were bagged, samples collected and measurements made throughout the day and night. Twenty flowers of different ages (1-4 days) were chosen and nectar was withdrawn from the base of the floral tube using a capillary tube on daily bases. Nectar samples were dried on Whatman No. 1 filter paper. The nectar sugar composition was analysed using basic HPLC technique (van Wyk 1993).

Field experiments were undertaken in Populations 1 and 7 to compare fruit set in the presence and absence of pollinators. Ten big plants which have not started flowering were selected in February 2002 and covered with shade netting in order to exclude pollinators before pollination commenced in Population 1. Ten other similar plants were selected and treated as controls. In Populations 7, five covered and five uncovered plants were selected from the annual burn (firebreak), two year burning cycle and four year burning cycle in January to February 2003. We also studied whether *B. greenii* is self-compatible (not done in the field). Pistils were self-pollinated with pollen from the same flower and cross-pollinated with pollen from another flower just before anthesis outside Great Hall at University of the Witwatersrand for 24 hours. Pistils were fixed in 3:1 ethanol:acetic acid for three hours, cleared in 8N NaOH for 60 minutes, rinsed with water, stained with aniline blue and mounted with DPX microscopic mountant. Pollen tube development in pistils was viewed under a Nikon SMZ 1500 light microscope connected to a Nikon Digital Camera DXM 2000. Stigma receptivity (also done with flowers outside Gate House at University of the Witwatersrand) was determined before anther dehiscence by applying hydrogen peroxide to the stigmas and viewing under a dissecting microscope in order to observe bubbles formation (indicating peroxidase activity and receptivity) (Kearns and Inouye 1993).

Allozyme variation and differentiation

Allozyme electrophoresis was initially done on three populations from the two years burning regime on privately owned land (Makholela et al., 2003). The attempt in the current study included three populations from the four years burn plan in the Nature Reserve. In addition five individuals from each of all known populations of *B. greenii* were analyzed to determine how different these populations were from each other.

Stratified sampling was used to cover the range of spatial distribution within populations. A tape measure was placed parallel to the long axis of the population. Sampling was done at more or less regular intervals along the tape in a zig-zag pattern across the full width of the population. Sampled individuals were marked with tags in case there is a need for further investigations.

Young leaves were collected from growing shoots, placed in cryotubes and immediately submerged in liquid nitrogen (-196EC). Leaf tissue extracts were prepared and analysed by starch gel electrophoresis using the extraction buffers, standard electrophoretic procedures, methods of interpretation of gel banding patterns and locus nomenclature followed by Van der Bank et al. (1995). Locus abbreviations, buffer systems used and enzyme commission numbers were the same as those used in Makholela et al. (2003).

The BIOSYS-2 computer program (Swofford et al., 1997) was used to calculate average heterozygosity (H), mean number of alleles per locus (A), percentage of polymorphic loci (P), Wright's fixation (F) indices and Nei's (1978) genetic distances (D) between populations.

Results

Red data assessment (IUCN Categories) and management regimes

Barleria greenii is very restricted at Estcourt with a very small area of occupancy (1.5 km^2) and fewer than ten locations (Table 1). It was on this basis that *B.greenii* has been given the conservation rating of Vulnerable (VU D2) in the current study. The species is also locally abundant where it is found (Table 1).

Table 1: Area of occupancy and number of individuals for known populations of *B. greenii*.

Pop.	Adults	Juveniles	Seedlings	Total	Area (hectares)	Density (ind/hectares)
1	14792	694	453	15939	26.019	612.590
2a	660	109	32	801	0.864	926.568
3	1263	109	32	1404	9.034	155.410
4a	1570	49	0	1619	0.735	2203.381
4b	4648	406	122	5176	13.226	391.332
4c	5	0	0	5	0.005	1004.016
2[a]	10333	359	99	10791	80.374	134.260
7[b]	5546	266	101	5913	7.393	799.811
6[a]	2720	38	21	2779	14.744	188.482
9	41	0	0	41	0.005	7663.551
Total	**41578**	**2030**	**860**	**44468**	**1.5240054**	

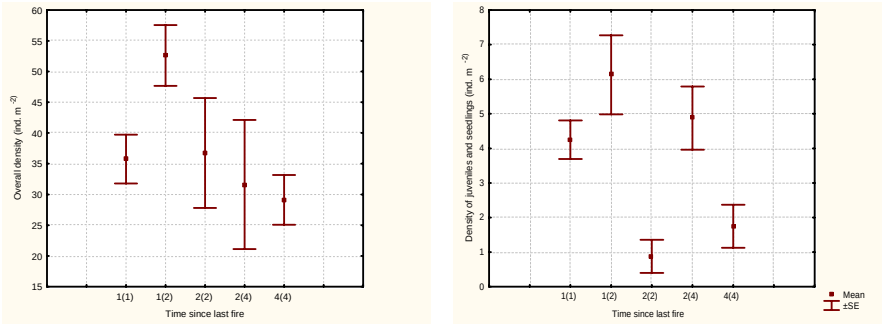
[a] Populations in the four year burning cycle
[b] Population with three different burning regimes (annual burn, two year burning cycle and four year burning cycle)

Density of individuals differed under different management regimes. Populations subjected to a two year burning cycle had a mean density (individual/hectare) of 1850.979 (n = 7) and were 11 times more denser than populations subjected to a four year burning cycle with a mean density of 161.371 (n = 2). High correlation between density based on total populations compared to density on a plot by plot basis was found ($r = 0.83$, $\rho = 0.08$).

The population numbering for management regime treatments was according to the time since fire and the length of the fire cycle and did not follow either Balkwill et al.

1990 numbering system or the numbering system in the current study. One year post fire populations (Population 2a and part of Population 7) in the two year burning cycle were written as 1(2) where 1 stands for the time since the last fire and 2 in parentheses for the length of the fire cycle. Therefore 1(1) indicates a population in the annual burn (part of Population 7), 2(2) for two years post fire population (Population 3) in the two year burning cycle, 2(4) for two years post fire population (Population 2a) in the four year burning cycle and 4(4) for four years post fire population (Population 6 and part of Population 7) in the four year burning cycle.

Populations from 1(2) were denser (Figure 2) for overall density and significantly different from 4(4) ($\rho = 0.001$), nearly significantly different from 1(1) ($\rho = 0.046$) and 2(4) ($\rho = 0.0.052$), and not significantly different from 2(2) ($\rho = 0.138$). The other treatments were not significantly different from each other. On the other hand, density of juveniles and seedlings was highest in 1(2) and significantly different from 2(2) ($\rho = 0.004$) and 4(4) ($\rho = 0.002$). 2(2) was also significantly different from 1(1) ($\rho = 0.0009$) and 2(4) ($\rho = 0.0003$) for density of juveniles and seedlings. In addition 1(1) was significantly different from 4(4) ($\rho = 0.016$) whereas 2(4) was significantly different from 4(4) ($\rho = 0.001$).



a b

Figure 2: Overall density (a) and density of juveniles and seedlings (b) in specific populations of *B. greenii* in which the effects of management regimes were studied, n = 8 in 1(1), 1(2), 2(4) and n = 16 in 2(2) and 4(4). x(x) = x is the time since fire and (x) is the length of the fire cycle.

Biomass of grass, litter and forbs also differed between the various burning regimes. Biomass in four years post fire in the four year burning cycle was highest and significantly differed from 1(1) ($\rho = 2.4 \times 10^{-8}$), 1(2) ($\rho = 2.25 \times 10^{-6}$), 2(2) ($\rho = 5.52 \times 10^{-6}$) and 2(4) ($\rho = 0.001$) (Figure 3). 1(1) on the other hand significantly differed from 1(2) ($\rho = 0.0004$), 2(2) ($\rho = 0.025$) and 2(4) ($\rho = 6.26 \times 10^{-5}$). In addition 2(2) was significantly different from 2(4) ($\rho = 0.020$)

Reproductive effort peaked in 2(4), tapers off slightly in 4(4) and 2(2) and was very low in 1(2) and 1(1) (Figure 4). Significant differences for reproductive effort were observed between 1(1) and 2(2) (ρ = 0.048), 1(1) and 2(4) (ρ = 0.005), 1(1) and 4(4) (ρ = 0.001), 1(2) and 2(2) (ρ = 0.013) and 1(2) and 4(4) (ρ = 0.0078)

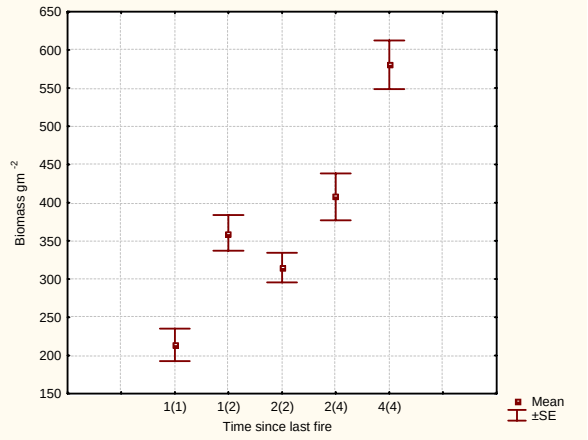


Figure 3: Biomass of grass, forbs and litter but excluding *B. greenii* in specific populations of *B. greenii*, n = 10 in 1(1), 1(2), 2(4) and n = 20 in 2(2) and 4(4).

Plant populations can be described as invasive, normal or senile (regressive) (Oostermeijer et al., 1994). The first type is characterized by high proportions of seedlings and juveniles relative to the adult stage. This is the opposite of the second (normal) type, where seedlings and juveniles will be present in the population but adult flowering individuals are in the highest proportion. In the third population type, seedlings and juveniles are totally absent. For *B. greenii*, two population types were distinguished: “normal type” in most populations and “senile type” (Figure 5) in small populations (Table 1). “Normal type” also dominated when similar calculations were done on a plot by plot basis.

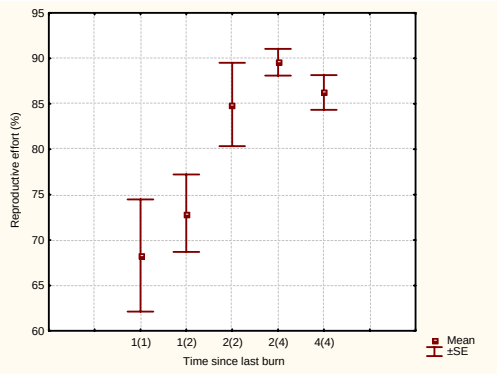
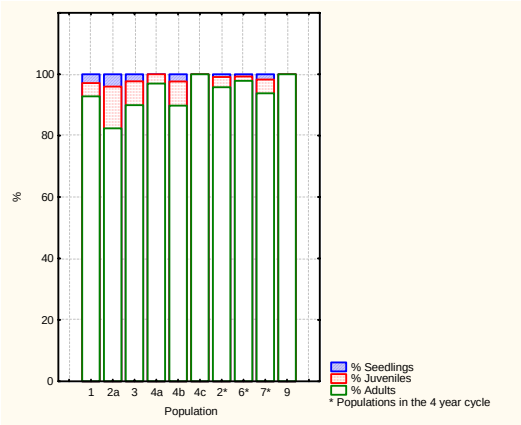
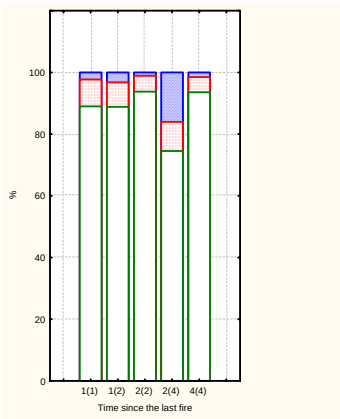


Figure 4: Reproductive effort in specific populations of *B. greenii* in which the effects of different management regimes were studied, n = 8 in 1(1), 1(2), 2(4) and n = 16 in 2(2) and 4(4).



a



b

Figure 5: Population composition in all populations of *B. greenii* (a) and selected *B. greenii* populations sampled on a plot by plot basis (b).

Canopy area and volume index reflected the same data pattern. Both were high (Figure 6 and Figure 7) in 4(4). However, there were some individuals with higher canopy areas and volume indices in the two year burning cycle (1(2) and 2(2)). These individuals were the ones in which the above ground parts (stems) were assumed to have survived fire.

Canopy area was more strongly correlated with height in 1(2) and 2(4) than in 4(4) and 1(1) had the highest correlation (Figure 8). Just as with canopy area and volume index, few large individuals were observed in the two year burning cycle, many large individuals were observed in 4(4) whereas the 1(1) and 2(4) comprised mainly small individuals (Figure 8).

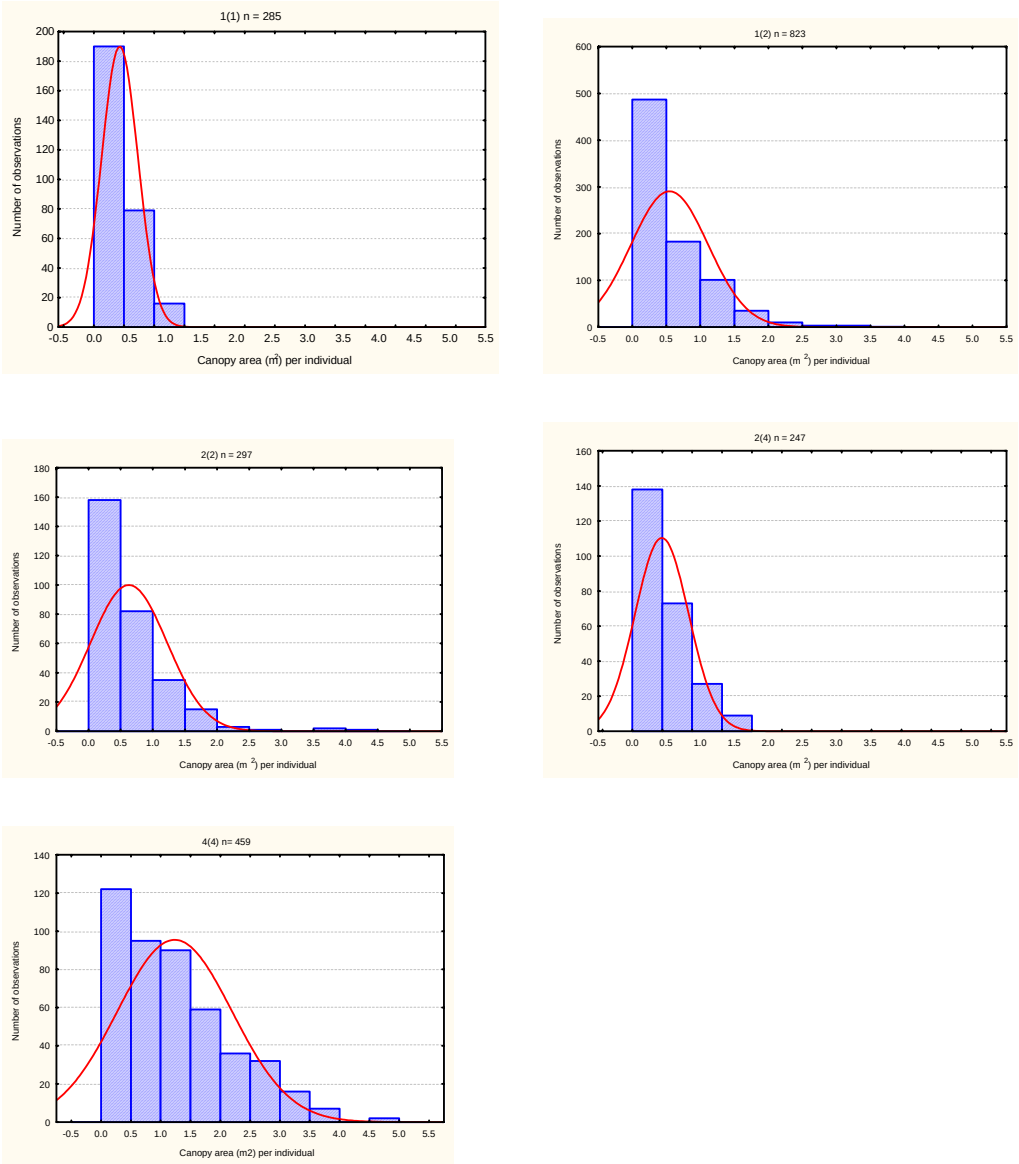


Figure 6: Histogram of canopy area distribution in selected populations of *B. greenii* in which effects of management regimes were studied.

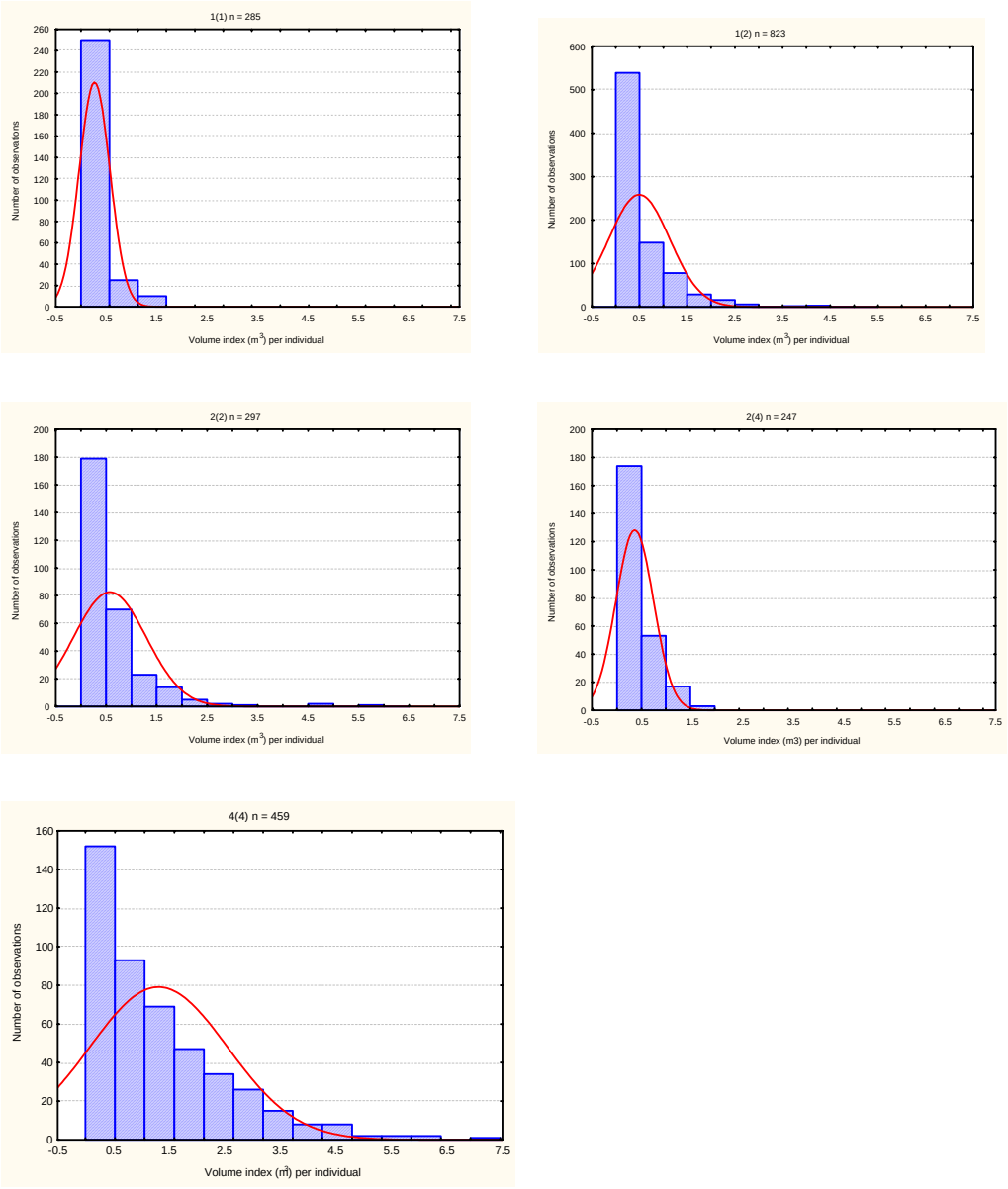


Figure 7: Histogram of volume index distribution in selected populations of *B. greenii* in which effects of management regimes were studied.

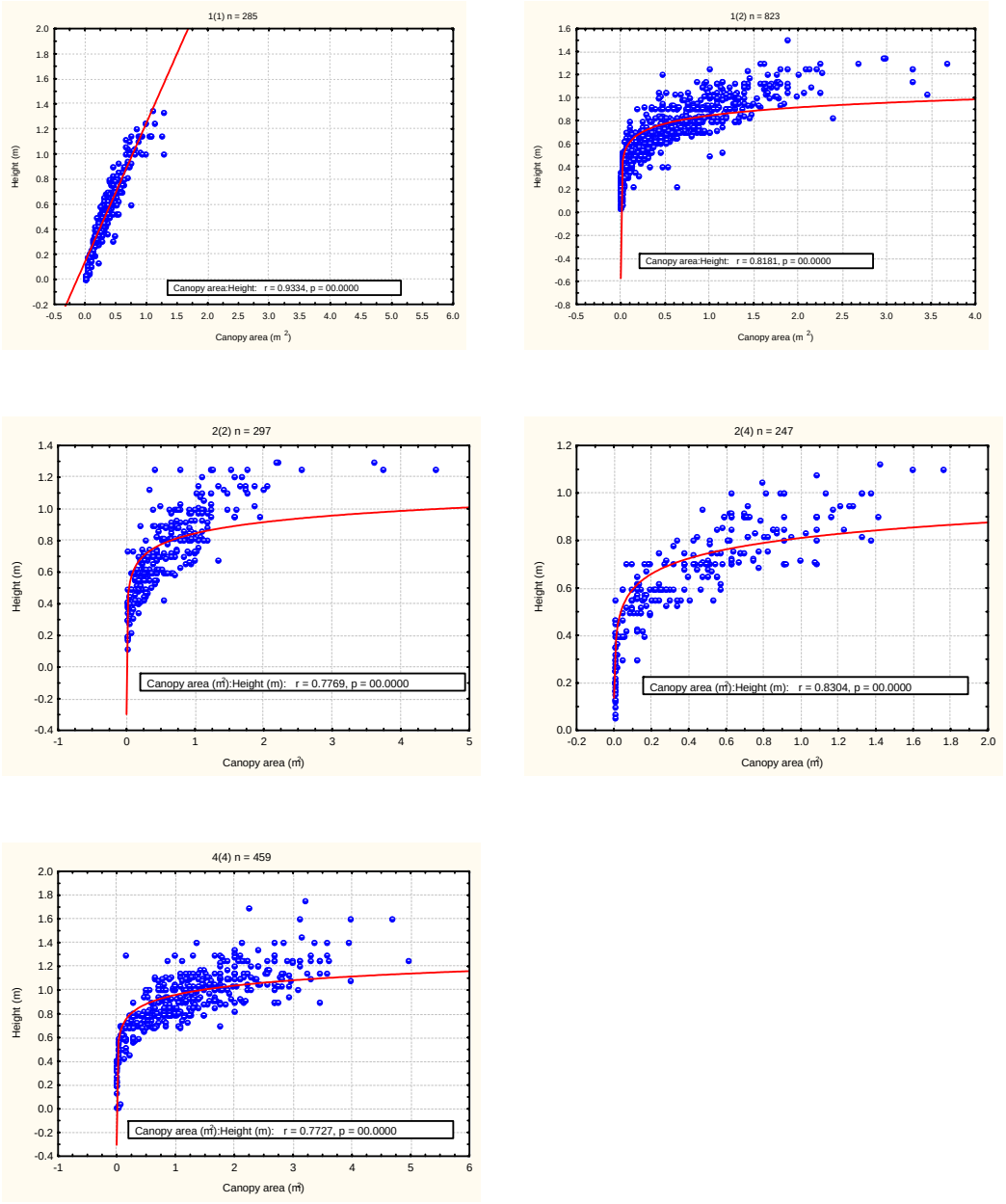


Figure 8: Correlations between canopy areas and heights in selected populations of *B. greenii*.

Reproductive biology, fruit set and nectar sugar composition

Flowers of *B. greenii* are scented and with colour ranging from white through light pink to dark pink. Plants with white flowers were rarely observed (< ten plants in each population) and were only found in Population 1 and Subpopulation 4a. The corollas are five-lobed with a long tube. Nectar is found at the base of the corolla tube. The superior ovary has four ovules. The ovary is protected by four calyx lobes; two inner small calyx lobes and two outer large calyx lobes subtended by bracteoles modified into spines. The androecium consists of two fertile lower and two fertile upper stamens. Filaments are permanently twisted and crossed-over near their bases (Balkwill et al., 1990). The anthers of the lower stamens are found at this crossover (Figure 9). The pollen grain size ranged from 82 to 132μm (n = 30). The stigma is held above the upper stamens.

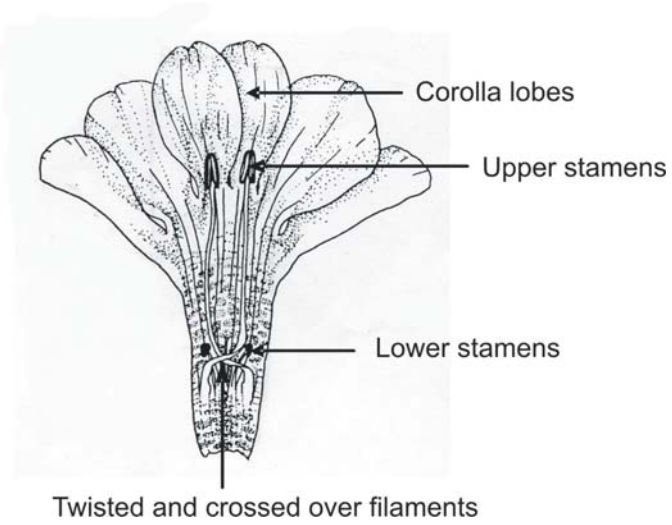


Figure 9: Twisted and crossed over filaments in *B. greenii*. Drawing done by Dr M. J. Balkwill.

Flower opening occurs at around 0600h to 0900h depending on weather conditions, i.e. flowers open earlier when it is hot than when it is cooler. The corolla lobes are the first to show the signs of anthesis by making a small opening in which the stigma can be seen above the upper stamens. Bubbles formation was observed in sixteen out of twenty flowers thus indicating that *B. greenii* flowers are protandrous. Anther dehiscence occurs 20 to 50 minutes after flower opening. By the time dehiscence is complete the corolla lobes have fully opened. Flowers can last for three to four days and corolla tube length increases from the first day to the last day whereas nectar volume decreases (Figure 10) from day 1 to 4. Nectar sugar composition was similar in different flower colours (white, light pink and dark pink) of *B. greenii* (Table 2). Nectars had more fructose and glucose than sucrose (Table 2).

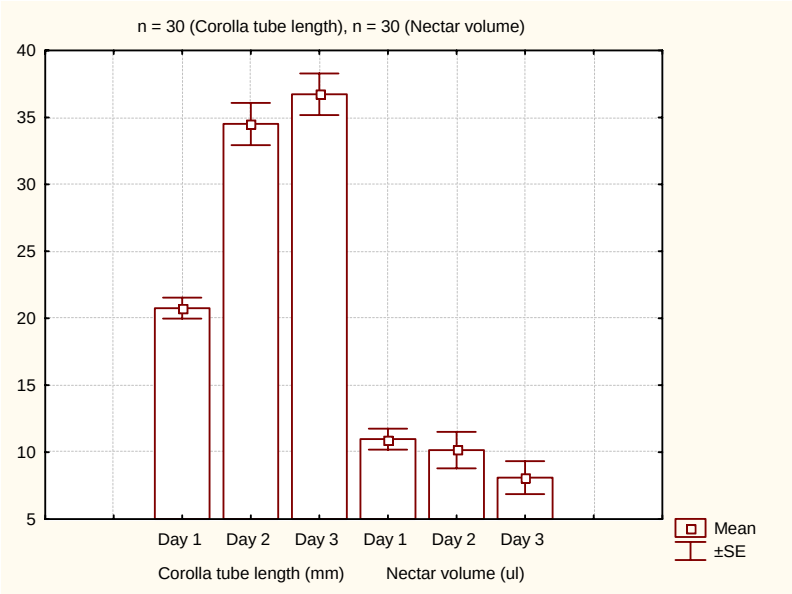


Figure 10: Increasing corolla tube length and decreasing nectar volume in *B. greenii* flowers from the first to the last day of their survival.

Three hawkmoth species (eight individuals), one other moth, two beetles, one carpenter bee and one unidentified caterpillar (Table 3) from Subpopulation 4a and Populations 2a and 3 were collected visiting the flowers of *B. greenii* from the field and brought to the laboratory for further examination (presence/absence of pollen grains, proboscis length, body mass etc (Table 3). Although these were the only visitors collected, carpenter bees were the most frequent visitors during the day whereas hawkmoths visited at night. Carpenter bees visited between 0800h and 1700h in order to suck nectar by making a slit in the corolla tube. 20, 8, 25 and 33 carpenter bees were observed for 4 consecutive days in Subpopulation 4a. Only 8 carpenter bees visited on day two because it was raining. Hawkmoths visited the flowers between 1800h and 2345h. Most hawkmoths frequently visited between 1800h and 1930h but only one observation was made at 2345h for four days. Nineteen hawkmoths were observed spending approximately 3.7±2.1 (mean±SD) seconds in each flower within a time frame of 30 minutes in Subpopulation 4a. The same numbers of hawkmoths were recorded in Population 3 in 1 hour 15 minutes spending 2.3±1.3 seconds in each flower. For Population 2a, 19 hawkmoths were recorded in 45 minutes. These hawkmoths spend 2.7±1.2 seconds in each flower.

The hawkmoths hovered briefly in front of the flowers before inserting their proboscis into the corolla tubes (Figure 11). They then probed into the corolla tube to the full extent of their proboscis. During this process their abdomen, legs and proboscis touched the anthers and stigma (Figure 12). It is assumed that a pollen-bearing hawkmoth visits another flower and inserts its proboscis into the corolla tube, brushes the sticky stigmatic surface and pollen grains attach to the stigma. Viewing captured

hawkmoths revealed quantities (Figure 13) of pollen, which were identified to correspond to that of *B. greenii* pollen (Figure 14) especially on the abdomen, legs, and very little along the proboscis. A third hawkmoth, *Agrius convolvuli*, had a longer proboscis and could suck nectar without touching the lower stamens. Viewing captured *A. convolvuli* revealed no pollen grains along the proboscis.

The moth was seen from 2000h to 0400h and stayed on the corolla lobes without touching the anthers and the stigma. A small beetle entered flowers through spaces between the corolla lobes and spent up to an hour on one flower. It removed nectar and pollen grains in one extended visit (Figure 15a and b). A relatively small and short tongued carpenter bee robbed nectar by making a small slit at the base of the corolla tube followed by nectar sucking with its short proboscis (Figure 15c). The caterpillar visited throughout the day, destroying entire parts of flowers, buds and fruits. Chewing damage to the calyces, filaments and ovary mark attacked flowers that survive to anthesis.

Table 2: Nectar sugar composition for *B. greenii* and other unpublished species data in the family Acanthaceae as analysed by Ben- Erik van Wyk.

Species	FRUCTOSE	GLUCOSE	SUCROSE	SUCROSE:HEXOSE
Butterfly pollinated				
Dicliptera clinopoda	50	48	2	0.020
Hypoestes aristata	25	73	2	0.020
Ruspolia hypocrateriformis	30	27	43	0.754
Butterfly/moth pollinated				
Dicliptera extenta	48	49	3	0.031
Bird pollinated				
Anisotes rogersii	50	50	0	0
Metarungia longistrobus	50	49	1	0.010
Ruttya fruticosa	50	50	0	0
Moth pollinated				
Barleria albostellata	26	18	56	1.273
Barleria pretoriensis	16	18	66	1.941
Barleria greenii (light pink)	51	47	2	0.020
Barleria greenii (deep pink)	50	48	2	0.020
Barleria greenii (white)	52	48	0	0
Bee pollinated				
**Barleria* sp. *cf. obtusa*	50	50	0	0
**Barleria obtusa* (dark purple)	50	50	0	0
**Barleria obtusa* (pale blue)	50	48	2	0.020
Duvernoia adhatodoides	10	10	80	4
Duvernoia aconitiflora	45	47.5	7.5	0.081
Asystasia gangetica	43	35	22	0.282
Unknown pollinators				
Ruttya ovata x Ruspolia hypocrateriformis	29	26	45	0.818
Barleria cf elegans (hybrid)	39	34	27	0.370
Barleria rotundifolia (yellow)	50	47	3	0.030

* Species belonging to the same Section of *Barleria* as *B. greenii.*

Table 3: Visitors to the flowers of *B. greenii*, their biomass, body length and proboscis length.

Visitors	**Biomass (g) (mean±SD)**	**Body length (mm) (mean±SD)**	**Proboscis length (mm) (mean±SD)**
Sphingidae (Hawkmoths)			
1. *Hippotion celerio* (n = 4)	0.7±0.115	38±4.62	34.5±0.173
2. *Nephele comma* (n = 1)	1.3	53	40.6
3. *Agrius convolvuli* (n = 3)	1.2±0.1	58±2.89	90.1±19.950
Eupterotidae (Moth)			
1. *Jana tantalus* (n = 1)	0.6	50	
Cetoniinae (Beetles)			
1. *Porphyronota hebreae* (n = 1)	0.23	23	Not applicable
2. *Crytothyrea rubriceps* (n = 1)	0.09	115	Not applicable
Anthophoridae (Carpenter bee)			
Xycolopa caffra (n = 1)	0.24	24	6.5
Somabrachidae (Caterpillar)			
Unidentified caterpillar (n = 1)			Not applicable

The species is protandrous with anthers and stigmas being spatially separated and flower diameter exceeding 6 mm thus giving *B. greenii* a Cruden Outcrossing Index of strongly outcrossing, possibly self-incompatible. P:O was 90:1 and is indicative of facultative autogamy. Pollen tube growth was observed in self-and cross-fertilized stigmas (Figure 16a and b). Anova revealed no significant difference ($\rho = 0.07$) in fruit set in plants covered with shade net and those which were not covered. In general fruit set was very low, out of 98.125±171.266 (mean±SD, n = 24) flowers (counted from the total number of calyces in each plant), only 4.458±6.541 (n = 24) set fruits in plants covered with shade net and only 12.667±20.626 (n = 24) out of 166.417±300.687 (n = 24) set fruits in uncovered plants.



Figure 11: *Nephele comma* with its proboscis uncoiled just prior to entering a corolla tube.

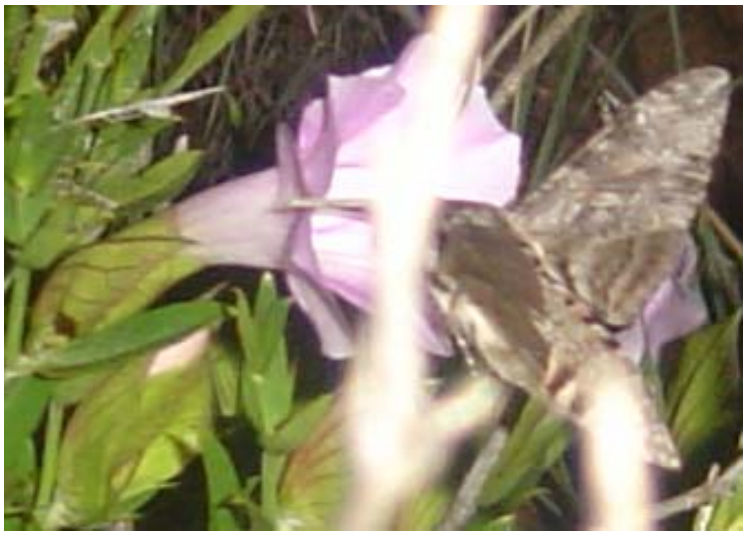


Figure 12: *Nephele comma* with its proboscis inserted inside a corolla tube.



Figure 13: Pollen grains on the abdomen and legs of *Hippotion celerio* (a, b) and proboscis of *Nephele comma* (c, d).

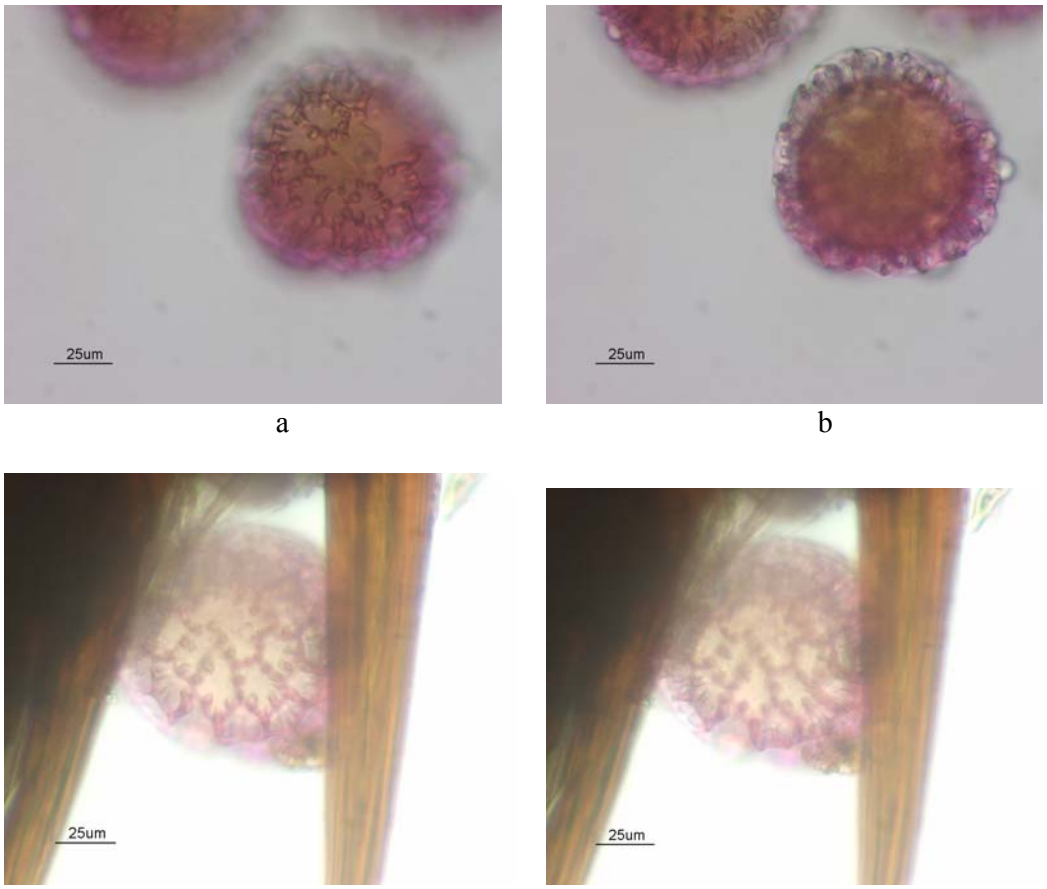


Figure 14: *Barleria greenii* pollen grains from Herbarium specimen collected by Dave Green (no. 499) and corresponding to pollen found on *Hippotion celerio* legs.



a

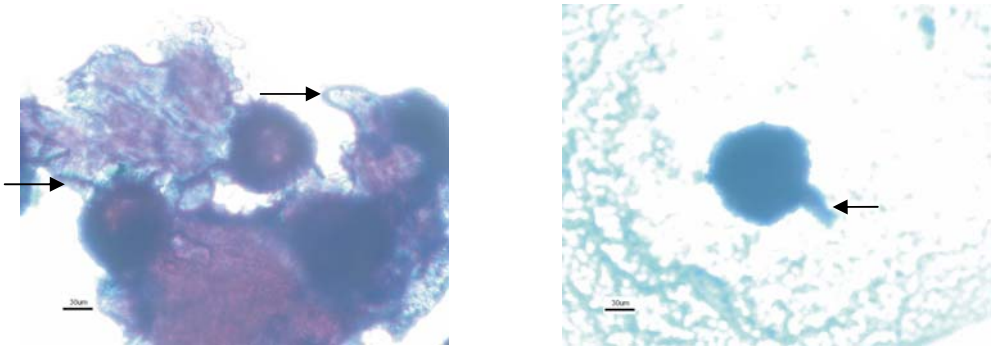


b



c

Figure 15: *Cytothyrea rubriceps* sucking nectar (a, b) at the base of a corolla tube and *Xylocopa caffra* making a slit in a corolla tube to suck nectar. Photographs taken by Professor Kevin Balkwill.



a b

Figure 16: Pollen tube growth on stigmas which were self pollinated (a) and cross pollinated (b) for 24 hours.

Allozyme variation and differentiation

Ten enzyme coding loci provided interpretable results in three analysed populations from the Weenen Game Reserve. Three loci (30%) were polymorphic and seven (70%) displayed monoalleleic gel banding patterns. Staining of some enzymes which provided interpretable results in the previous chapter (Chapter 2) did not give any results in this study. These were AAT and EST. Enzymes which were polymorphic (PEP-E and GPI-2) in populations in the Weenen Game Reserve were also polymorphic in the previous study. GPI-1 was polymorphic in this study but not in the previous study.

Deviations of genotypic distribution from Hardy-Weinberg expectations occurred at all loci. No genotypes closely approximated the Hardy-Weinberg expectations. Deficiencies of heterozygotes occurred at all three polymorphic loci (PEP-E in Population 7, GPI-2 and PEP-E in Populations 2a and 6). The A, P, and H values are presented in Table 5 and were very low. However, Population 2 had the highest A, P and H compared to the other two populations.

Table 5: Mean number of alleles per locus (A), percentage of polymorphic loci (P) and average heterozygosity (H).

Population	**A**	**P (%)**	**H**
1[a]	1.19	18.75	0.067
2a[a]	1.44	31.25	0.500
3[a]	1.50	43.75	0.086
2[b]	1.20	20.00	0.026
7[b]	1.10	10.00	0.019
6[b]	1.10	10.00	0.010

[a] Populations analysed in the previous chapter (Chapter 2) and from the privately owned land (two year burning cycle)

[b] Populations analysed in the present study and from the Weenen Game Reserve (four year burning cycle)

Values of D, F_{IS}, F_{IT}, F_{ST} and N_{em} are presented in Table 6 (see previous chapter for definitions). The low D values between populations suggest little differentiation. High F_{IT} and F_{IS} values are indicative of high inbreeding rates. As with D values, the F_{ST} values are indicative of little differentiation, i.e. 96.5, 99.5 and 95.4% of the differentiation was within population compared to 0.5-4.6% between populations. The pair-wise contingency χ^2 analyses at all loci indicated a significant (ρ = 0.041) difference in mean allelic distribution between Populations 6 and 7. The locus that contributed most to population differentiation was PEP-E with a ρ value of 0.035. A significant differences were also observed between Populations 2a and 6 (ρ value of 0.047), with PEP-E (ρ = 0.050) contributing most to population differentiation. No significant difference was observed between Populations 2a and 7. The high N_{em} values between all populations suggest substantial gene flow between populations with gene flow being highest between Populations 2a and 7. No population differences, in terms of genetic variation and differentiation, were detected when five individuals from each of the known populations of *B. greenii* were analysed except for one individual with rare alleles in Populations 2a and 6 and two individuals in Population 9. With such a small sample it is impossible to draw any valid conclusions with regard to Population 9.

No significant correlations were found between area of occupancy and A (r = -0.2828, ρ = 0.5871), P (r = -0.1803, ρ = 0.7324) or H (r = -0.3962, ρ = 0.4368). Moreover no significant correlations were found between population size (number of individuals per population) and A (r = -0.4791, ρ = 0.3363), P (r = -0.3831, ρ = 0.4535) or H (r = -0.4278, ρ = 0.3975) values.

Table 6: Nei's (1978) genetic distance (D), geographical distance, summary of F-statistics for all polymorphic loci and the effective number of individuals exchanged in each generation (N_{em}).

Populations	**D**	**Distance**	**F_{IS}**	**F_{IT}**	**F_{ST}**	**N_{em}**
1 and 2a	0.008	3.24	0.605	0.635	0.076	1.95
1 and 3	0.006	4.87	0.605	0.626	0.054	1.35
2a and 3	<0.001	1.91	0.611	0.617	0.015	7.30
2 and 7	<0.001	2.65	1.000	1.000	0.005	21.68
2 and 6	0.001	3.34	1.000	1.000	0.035	3.08
6 and 7	0.001	1.77	1.000	1.000	0.046	2.30

The matrix of the pairwise genetic distance was not significantly correlated with the geographical distance (r = 0.6524, ρ = 0.1602). In addition no significant correlation was found between the average numbers of individuals exchanged between populations per generation (N_{em}) and geographical distance (r = -0.2890, ρ = 0.3890).

Discussion

Red data assessment (IUCN Categories) and management regimes

Barleria greenii was initially given the conservation rating of Vulnerable (Hilton-Taylor 1996). The rating by Hilton-Taylor lacked information on area of occupancy, extent of occurrence and it underestimated the total number of individuals. Rarity in *B. greenii* is a result of a small distribution area on a global scale. Its distribution and local abundance is restricted by habitat constraints i.e. occurrence in doleritic soils. Few plant species (4%) have been observed to depend on the presence of particular rare habitat types (Pärtel et al., 2005). The persistence of specific natural habitat types is mostly dependent on restrictive regulations. Consequently, these habitat types should be strongly protected *in situ* (Pärtel et al., 2005). Populations of *B. greenii* are well protected at the moment falling either within the Lowlands East Conservancy or the Weenen Nature. However, there is a road passing through Population 3. Therefore consideration should be given to re-routing this road.

Densities of populations in the two year burning cycle significantly differed and exceeded that of populations in the four year burning cycle. Populations in the four year burning cycle were less dense but with more biomass. Consequently, seedling germination and recruitment is limited by the presence of plants, i.e. more biomass, other than *B. greenii* as a result of infrequent burning and low grazing. Large differences in fire response among plant communities have been observed (Brys et al., 2005). High rates of biomass production and litter accumulation may lead to shorter time intervals between subsequent fires and a higher fire severity, both of which may negatively affect germination, survival rates in the dormant seed bank and plant growth after fire of residing plant species (Brys et al., 2005).

Although normal population type dominated most populations, the density, biomass, canopy area and volume index differed depending on management regime. Populations in the two year burning cycle were denser with less biomass and had many small individuals and few large individuals (Figure 17 and 18). Populations in the four year burning cycle were less dense with more biomass and constituted mainly large individuals (Figure 17 and 18). The two years post fire population in the two year burning cycle was less dense and had less biomass compared to one year post fire in the same burning cycle. The habitat in this population was rocky thus limiting open sites for *B. greenii* colonization. Therefore, not only the population type is important in population dynamics of *B. greenii* but a combination of factors (density,

canopy area, management regime etc) provided a better understanding of the population dynamics of this species.

Reproductive effort differed depending on the time since the last fire. During the first year after fire, plants invest more on replenishing vegetative resources hence the reason for the low and significantly different reproductive effort compared to other treatments (2(2), 2(4), and 4(4)). High reproductive effort in 2(2), 2(4), and 4(4) (Figure 4) is likely a result of the plants in these populations having rebuilt underground resources and mobilising those resources to replenish both vegetative and reproductive above ground parts. Although there is more reproductive effort during the second year after fire in the four year burning cycle, it does not benefit *B. greenii* since from the second year after fire in the four year burning cycle, the biomass of other plants likely outcompetes *B. greenii* seedlings for resources i.e. light and water.

Although different management regimes used in populations of *B. greenii* might not have benefited *B. greenii* (since it was not known as to which management regime was suitable for *B. greenii*), it has provided an opportunity to assess which is the more appropriate strategy. The two year burning cycle with moderate grazing was found to be the most appropriate management regime for *B. greenii* because plants from populations in the two year burning cycle were more denser and by the second year after fire, these plants can invest more on reproductive effort just as with plants from the four year burning cycle (Figure 4). All populations in the two year burning cycle are going to be part of the Gongolo Reserve. Associated with the establishment of the reserve will be management strategies, which might be different from the current strategies that best suit *B. greenii*. Therefore with the results from this study, an active uniform management regime can be adopted when the Gongolo Reserve is established.

In addition to low density and few open sites for *B. greenii* colonization, populations in the Weenen Game Reserve (four year burning cycle) had less genetic diversity and high heterozygote deficiencies compared to populations in the privately owned land (two year burning cycle) (see chapter 2 and the next section on allozymes). This is probably because in two year burning cycle cool fires which destroy above ground parts but not below ground parts of juveniles and seedlings are produced. These juveniles and seedlings can then resprout after fire and, if they had any rare alleles, these alleles can be passed on to the next generation (by pollination) thus increasing genetic variation/differentiation. In the four year burning cycle, the intense fires can destroy both the above and below ground parts of seedlings and juveniles thus destroying any genetic variation/differentiation. Therefore, unless the juveniles in the four year burning cycle can survive fire by chance, the genetic variation in

populations in the four year burning cycle would always resemble that of founder individuals.

The low genetic variation can also be linked to density of individuals. The low density of individuals in the populations in the four year burning cycle likely limits the chances of finding rare alleles in these populations whereas in the two year burning cycle the high density of individuals increases chances of finding rare alleles hence the likely reason for more genetic variation in the two year burning cycle.

Reproductive biology, fruit set and nectar sugar composition

Filament characters have been recognized to be of significant value in pollination in the family Acanthaceae (Balkwill et al., 1990; Manktelow 2000). The filaments may be synorganized, dividing the corolla into two longitudinal compartments (filament curtain) (Manktelow 2000) or may be twisted and crossed over (Balkwill et al., 1990). The evolutionary origin of the filament curtain is probably concerned with its functions in pollination biology which are proposed to be restriction of nectar access, prevention of nectar evaporation, lever arm function and facilitating dorsal pollen deposition and stabilizing of posterior position of anthers and style (Manktelow 2000). *B. greenii* does not have the filament curtain but the crossed-over filaments function the same way as the filament curtain by restricting nectar access to certain species. Pollinators in *B. greenii* (hawkmoths) do not have to force the crossed-over filaments open due to the broad corolla tube of the flowers and their long proboscis. *Xylocopa caffra* can also insert its short proboscis into the broad corolla tube but cannot gain access to nectar due to its short proboscis. It thus makes a slit in the corolla tube in order to rob flowers. *Crytothyrea rubriceps* was able to push itself against the crossed-over filaments because of its small body size (Table 4), hence it is likely that pollen grains from the lower stamens can be deposited on its body.

The relative effectiveness of each hawkmoth species as a pollinator depends largely on the relative measurements of the flower and the insect (Johnson 1994, Johnson and Litveld 1997, Manning and Snijman, 2002). A long corolla tube (more than 30 mm long) is especially characteristic of flowers specialized for pollination by hawkmoths (Sphingidae) (Knudsen and Tollsten, 1993). The lengths of the proboscis of *H. celerio* and *N. comma* closely resemble the corolla tube length of *B. greenii* flowers and these hawkmoths must fully insert their proboscis to gain nectar. In doing so, their abdomens and legs touch the anthers and stigmas and pollen grains attach to their legs and abdomens. The long proboscis of A. *convolvuli* restricts it from making contact with the upper stamens. Although no pollen grains were found on *A. convolvuli* it is possible that the lower stamens might deposit pollen grains on its proboscis. These pollen grains could then be deposited on the stigma while the hawkmoth inserts its proboscis into another flower.

Although *B. greenii* is pollinated by hawkmoths its sucrose proportion (0-3%) is lower than that reported by Baker and Baker (1983) for typically moth pollinated flowers (over 50%). Following the classification by Baker and Baker (1983), the nectar sugar composition of *B. greenii* is classified as being characteristic of flowers pollinated by bats and flies. In comparing *B. greenii* with other species in which nectar sugar composition data are available no consistency was observed between nectar sugar composition and pollination types except for species pollinated by birds (Table 2). Nectar sugar composition, was however of taxonomic value, because sucrose composition for most species in the genus *Barleria*, except *B. albostellata* and *B. pretorienses*, was less than 50% (0 – 37%). The same was true for species in the genus *Dicliptera*. This consistency was initially reported by van Wyk (2002) at the South African Association of Botanists Conference at Rhodes University in Grahamstown.

Tracking pollen movement showed that there is a mixed mating system in *B. greenii* whereas indirect measurements showed no barriers to gene flow in populations of *B. greenii*. It is assumed that the hawkmoths can move between most populations of *B. greenii* in one night although the exact flight distance is unknown. If there is a mixed mating system, pollen tube growth in self- and cross-fertilized stigmas, fruit set in the absence and presence of pollinators, no barrier to gene flow and pollinators are not scarce then why are there low levels of genetic variation in *B. greenii*. Two possible explanations can be attributed to these: founder individuals with very low levels of genetic variation and the absence of a self-incompatibility mechanism.

Allozyme diversity and differentiation

The genetic variability and differentiation in populations in the Weenen Game Reserve is less than that of populations in the privately owned land. The average genetic diversity in these populations is also less than reported values for endemic populations (Hamrick and Godt 1989). Several factors, including a founder effect at the time of colonization, selfing as well as habitat specialization combined with local adaptation may have contributed to the lack of genetic variation in *B. greenii*.

Breeding system is strongly expected to influence genetic structure of plant populations (Ehlers et al., 2002). In the present study the breeding system is facultative autogamy with hawkmoths as pollinators. It is possible that these hawkmoths can move between populations to exchange pollen. But since there are few rare alleles in each population the transfer of genetic material by hawkmoths might not aid much in increasing genetic diversity.

In accordance with Oostermeijer et al. (1994) and Schmidt and Jensen (2000), no correlation between population size and genetic diversity within the studied populations was observed. Contrasting results have been observed in other studies (Van Treuren et al. 1991, Raijmann et al. 1994, Travis, et al. 1996, Fischer and Matthies 1998).

Conservation implications

The findings in this study confirmed Hilton-Taylor's classification and have included detailed information on area of occupancy, extent of occurrence and the total number of individuals. *B. greenii* is globally threatened with low levels of genetic diversity, a mating system that facilitates inbreeding and varying recruitment and growth under different burning and grazing regimes. Thus it is important that the management of the Gongolo Nature Reserve takes into account the vulnerability of this species when devising management strategies for the area.

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