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# The consequences of monoecy and dioecy in congeneric species, and their implications for the evolution of dioecy in the genus Viscum

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The orientation of the ring at metaphase is critical, since only one orientation can result in the chromosomes segregating in such a way that the gametes would have a full compliment of genes. All other orientations result in gametes with duplications or deficiencies (Stebbins 1971). In order to produce viable gametes, the chromosomes of the ring must assume a zig-zag orientation across the equator such that each alternate chromosome in the ring migrates to the same pole. Other orientations at metaphase result in adjacent chromosomes migrating together and gametes having one duplicated segment and one missing segment, therefore being inviable (Garber 1972). Fig. 1.4 illustrates the possible orientations of translocation complexes across the equator.

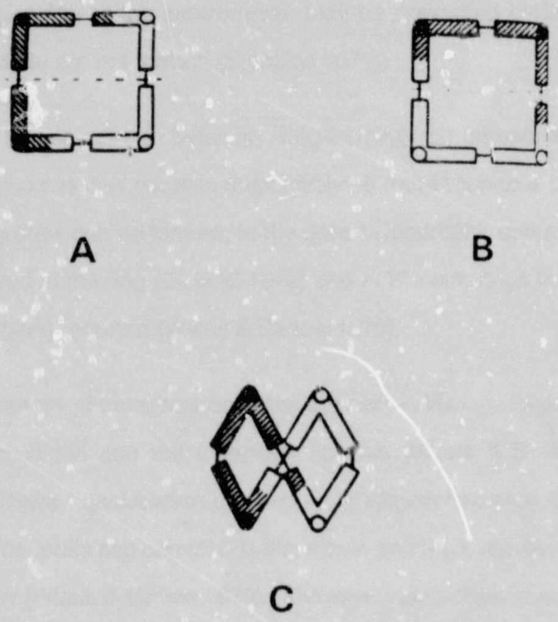


Fig 1.4. (A) & (B) Both pairs of chromosomes on either side of the equator (dotted line) have one duplicated segment and one missing segment. (C) Both pairs of chromosomes on either side of the equator have a full compliment of genes. There are no deficiencies and no duplications.

Since the only viable gametes will be those which received alternate chromosomes from the metaphase ring, it means that each gamete receives one of the two combinations of centromeres which were present in the parents of the structural heterozygote (Stebbins 1971). As a consequence, the genes that lie close enough to the centromere so that crossing over is unlikely, are not only linked to each other, but also to the genes lying close to the centromere of the other non-homologous chromosome involved in the translocation (Stebbins 1971). Chiasmata formation in these cases must be confined to the chromosome ends, so that the ring configurations are flexible to the extent that they can easily form the zig-zag shape so that alternate chromosomes assort together (Stebbins 1971). This implies that larger areas proximal to the centromeres may be protected from crossing over so that larger linkage groups are formed. Crossing over in the region of the centromeres may be prevented by large heterochromatic regions where chiasmata are not formed (Stebbins 1971).

Obviously, further translocations between ring-forming chromosomes and other non-homologous chromosomes can result in rings involving more than four chromosomes so that even larger linkage groups can be formed. In the genus *Oenothera*, some species have all their chromosomes involved in the ring (Cleland 1972) and in *Viscum*, rings involving 4, 6, 8 and 10 chromosomes have been reported (Wiens & Barlow 1979).

Wiens and Barlow's survey of translocation heterozygosity in *Viscum* revealed a strong association between this condition and the dioecious species (Wiens & Barlow 1979). The only monoecious taxa to have translocation complexes is *Viscum capense* ssp *hoolei*. The other subspecies, *Viscum capense* ssp *capense* is dioecious, and thus, ssp *hoolei* is believed to be a reversion to monoecy (Wiens & Barlow 1979). The expression of monoecy in *Viscum capense* ssp *hoolei* is altered, with some plants producing predominantly female flowers and others predominantly male flowers with few fruits maturing (Wiens & Barlow 1979). Each cymule in the inflorescence of the other monoecious species typically contains both male and female flowers, with the central flower usually staminate and the lateral flowers pistilate (Wiens & Barlow 1979 and Wiens & Tolken 1979).

Not only is there an association between dioecy and translocation complexes, but Wiens and Barlow (1979) believe their results indicate the presence of at least one additional translocation complex in the males of each dioecious species that is not present in the females of the species. They refer to these complexes as sex-associated translocation complexes as opposed to the

floating translocation complexes which occur in both sexes. The occurrence of sex-associated rings in dioecious species led them to suggest that these translocations are associated with the origin of dioecy in *Viscum* and they have presented a model whereby non-allelic male and female determining factors are brought into linkage by sex-associated translocations and thus serve to stabilise dicecy (Barlow et al 1978, Barlow 1981, Barlow & Wiens 1976 and Wiens & Barlow 1979).

It has been suggested that in the dioecious angiosperms, genes for maleness and femaleness are non-allelic (Ross & Weir 1975 and Charlesworth & Charlesworth 1978). If these genes were not linked, crossing over would continually result in hermaphrodites and neuters appearing, as well as males and females (Charlesworth & Charlesworth 1978). This would make it unlikely that unlinked genes for dioecy would ever be selected. Ideally, genes for dioecy should occur on the same chromosome in such a way that no crossing over could occur between the loci (Charlesworth & Charlesworth 1978). In *Viscum*, translocations could effectively be used to link the non-allelic sex factors. Fig 1.5 shows how male and female factors could be linked either by a single translocation, to produce a ring of four, or indirectly by two translocations, producing a ring of six.

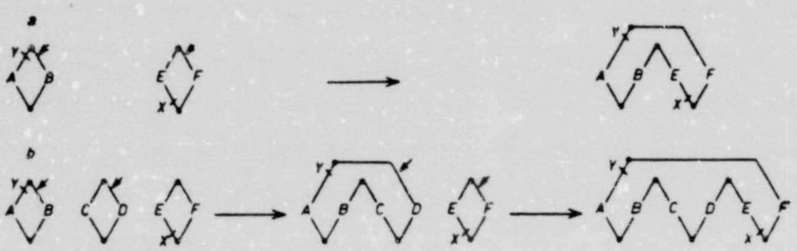


Fig 1.5. Y and X are the hypothetical sites of non-allelic factors for maleness and femaleness respectively. Possible breakage point are indicated by arrows. (a) Formation of a sex-associated ring of 4 by an interchange involving chromosomes carring the sex factors. (b) Formation of a sex-associated ring of 6 by interchanges linking X and Y indirectly through two different interchanges (After Wiens & Barlow 1979).

Although this is the cytological mechanism by which they propose the origin of dioecy in *Viscum*, Wiens and Barlow suggest, by quoting Bawa and Opler (1975), that dioecy in this case most probably arose in response to selection for the enforcement of outcrossing (Wiens & Barlow

1979). Wiens and Barlow (1979) mention the following hypotheses and state that possibly all of these factors may have contributed to the evolution of dioecy in *Viscum*.

1. The genetic simplicity of dioecy compared with self-incompatibility (Baker 1967).

2. High levels of reproductive failure in self-incompatible hermaphroditic or monoecious species because of the small foraging ranges of the pollen vectors.

3. Escape from seed predation caused by the altered size of seed crops and the distribution of seed-bearing plants.

(Bawa & Opler 1975).

Wiens and Barlow (1979) add that translocations may be of further adaptive importance in the genus as a means of maintaining high levels of heterozygosity.

# CHAPTER 2

# MATERIALS AND METHODS

## STUDY SITES

The main study site for this investigation was at Makapansgat, a farm near Potgietersrus in the central Transvaal. The populations investigated occur in a valley on the farm known as the Makapan Cave Valley or Makapansgat. There is a sub-perennial stream flowing through the valley, which includes areas of relict evergreen forest and part-deciduous fringing forest which are almost completely protected (Peters & Maguire 1981). The vegetation in the valley is by no means characteristic of the vegetation in the surrounding areas, which range from Acocks' North-Eastern Mountain Sourveld at the higher altitudes, to Mixed Bushveld at lower levels (Acocks 1953 and Peters & Maguire 1981). The bulk of the data collected were obtained from a population of *Viscum capense* ssp *capense* and a population of *Viscum rotundifolium* which occur sympatrically on this farm. It was felt that by studying the two species at one locality, site-specific environmental conditions which could affect the results would be minimised. Thus even if conditions were unusual during the year in which the study was undertaken, or had been stressful in preceding seasons, the results obtained for the two species would still be comparable. There are, however, limitations in drawing evolutionary conclusions from data collected for a single population of each species in a single season, so the ecological and reproductive findings should be viewed in this light. The genetic structure of the populations of each species is however not subject to the chance events of a single season and the genetic results can, therefore, be interpreted with a higher degree of confidence.

For the purpose of confirming results obtained in the genetic study of this section, additional populations from other sites were sampled. Apart from the collections made at Makapansgat, data were obtained for populations of *Viscum rotundifolium* from Nylsvley, the Magaliesberg and Grahamstown. Populations of *Viscum capense* ssp *capense* and *Viscum capense* ssp *hoolei* from Grahamstown were also investigated. Fig 2.1 shows the approximate localities of each site.

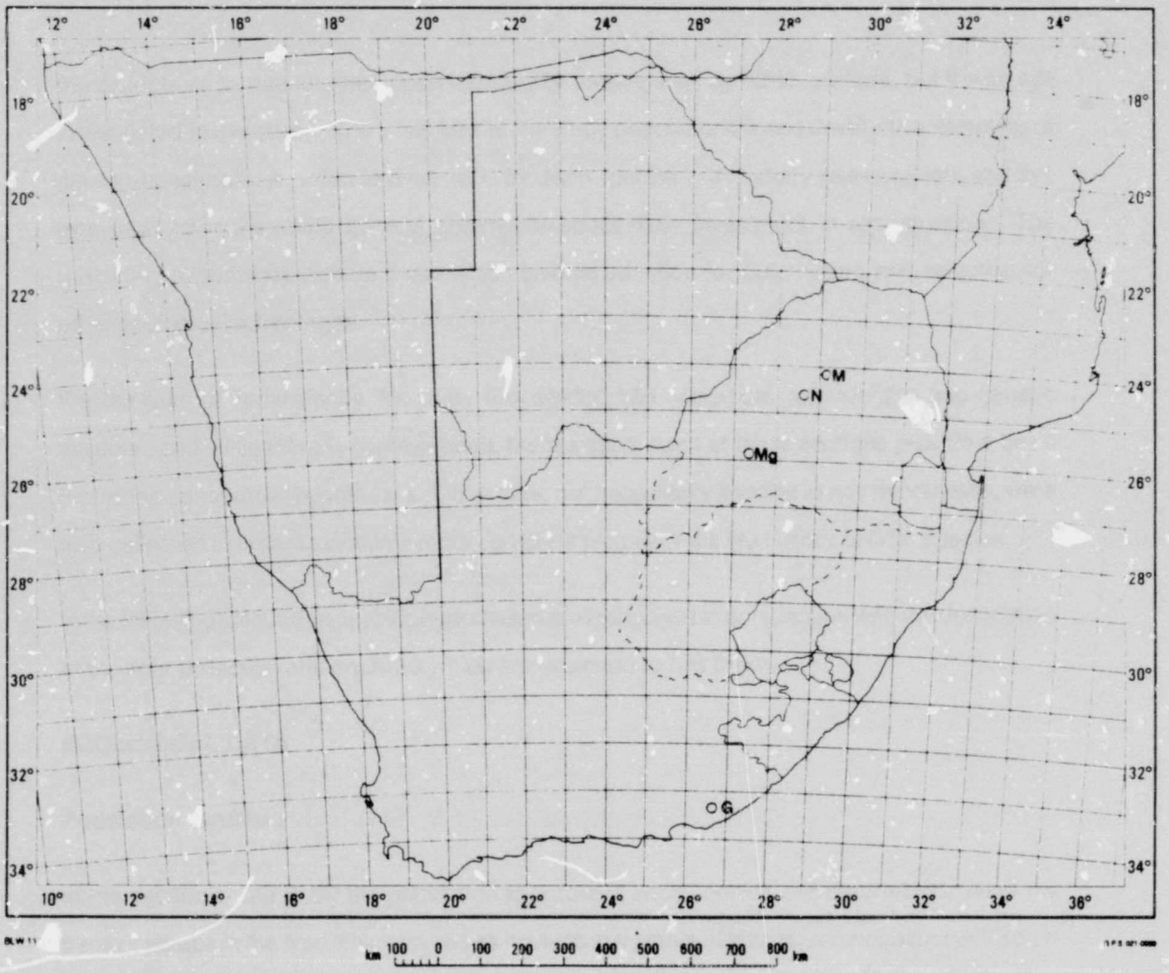


Fig 2.1. The localities of populations investigated in this study. (M) = Makapansgat; (N) = Nylsvley; (Mg) = Magaliesberg and (G) = Grahamstown.

## DATA COLLECTION

### INTRODUCTION

Since the aim of the study was to compare the two species' relative advantages and disadvantages with respect to several hypotheses for the evolution of dioecy, data were collected in such a way that the relevance of specific hypotheses to the evolution of dioecy could be tested. Obviously the choice of *Viscum* species for this study immediately excludes the hypothesis that dioecy could evolve to prevent ovule damage by pollinators (Givnish 1982), since the non-dioecious species in this study is monoecious, with unisexual flowers. Resource allocation is one of

the aspects of dioecy that has been thoroughly investigated by other workers, but it was not investigated in this study. The small size of the study populations made destructive sampling of plants undesirable. A pollen to ovule ratio for each species in this study was obtained, and this was used to make some general comments about male investment in reproduction. The remaining hypotheses include those which involve selection for outcrossing and selection for other ecological advantages.

For the sake of convenience this study was divided into ecological, reproductive and genetic sections, and while there is some overlap, the results of each of these sections provide a basis for testing some of the hypotheses. Other data, not necessarily specific to any hypotheses, were also collected in order to obtain a more complete picture of the life history of both species.

In the following discussion specific hypotheses are mentioned in conjunction with the description of the data collection and analyses which are designed to test them.

## ECOLOGICAL DATA

### Population structure

In this section of the study the population structure of each species was determined. All of the trees or shrubs in the area which served as hosts to any plants of *Viscum rotundifolium* or *Viscum capense* were labelled and identified. The numbers of plants in each host and their sex, in the case of *Viscum capense*, were then determined. Juveniles of *Viscum capense* were excluded from these counts since they are impossible to sex until they have flowered for the first time. By comparing the overall numbers of male and female plants in each of the host species, it was possible to determine whether the males and females of *Viscum capense* were favouring different host species, which could indicate selection for decreased intraspecific competition or disruptive selection in a patchy environment.

### Associations between plants

Measurements were made between randomly selected plants and the nearest plant of the same species for both *Viscum rotundifolium* and *Viscum capense*. The mean between-plant distance and standard deviation for each species was calculated. For *Viscum capense*, measurements were also made to test associations between sexes. Measures of distance were made between:

(a) Randomly selected males and the closest male

(b) Randomly selected females and the closest female

(c) Randomly selected males or females and the closest plant of the opposite sex.

For each of these three data sets the mean plant-to-plant distance and standard deviation were determined. In all cases, measurements were made with a tape measure between the closest points of each plant. Whenever branches of two plants were intertwined the distance between them was recorded as zero.

The measurements for *Viscum capense* were then used as an additional test for disruptive selection and decreased intraspecific competition. If disruptive selection had been in operation in *Viscum capense*, males and females would be expected to adapt to the patches where they performed the best, thus maximising the available resources and minimising intersexual or intraspecific competition (Freeman et al 1976). This would result in a spatial segregation of the sexes and females would be more highly associated with females than they would with males and vice versa. The mean plant-to-plant distance determined for *Viscum capense* was, therefore, compared with the mean male-to-male, female-to-female and the mean distance between plants of opposite sexes. If the mean male-to-male or female-to-female measures were smaller then the mean plant to plant measures for the species, it would indicate that the separate sexes are highly associated with themselves and that disruptive selection, or selection to minimise intersexual competition, may have taken place. This could also be inferred if the mean measure between plants of opposite sexes was larger than the mean plant to plant distance.

**Aggregation of species**

In order to complete the data on the population structure of both species, the degree of clumping of the plants was determined using the method of Clark and Evans (1954). This method was chosen since it uses nearest-neighbour measures which were already necessary for the study. Alternative methods require laying of quadrats, which is obviously inappropriate to a study of aerial parasites. Methods involving quadrats, or even line transects, operate in only two dimensions and would, therefore, have eliminated the vertical component of distance between the mistletoes and thus altered the results. Nearest-neighbour measurements made with a tape measure accurately record the between-plant distances of these populations, even though their

plants are distributed in three dimensions.

The method of Clark and Evans', involves the calculation of R, which is the ratio of the mean nearest-neighbour distance (or mean observed distance, Ro), to the distance expected from the density of the plants within the study site (Re). The mean plant to plant distance had already been determined above and density (P) is calculated by dividing the total number of plants in the population (N) by the area that the population is distributed over (A), as shown below.

$$P = \frac{N}{A}$$

The distance expected between plants based on the density of the population, is calculated as follows:

$$Re = \frac{1}{2\sqrt{P}}$$

An R value equal to 1 indicates that the population is distributed in a random fashion. Any value between 0 and 1 indicates aggregation and the higher the degree of aggregation, the closer the R value lies to 0. If the population is dispersed regularly, R lies between 1 and 2,1496, at which point all the plants would be equidistant from each other (Clark & Evans 1954).

This method is included in the ecological section since it is pertinent to the population structure. The degree of clumping is, however, important in making some inferences about dispersal, and will be discussed further in the reproductive section. In this study clumping has also been used to investigate the hypothesis that distribution of seeds in dioecious populations may aid in reducing seed predation, but this is also dealt with in the reproductive section.

Finally, the degree of clumping of the host species of *Viscum rotundifolium* was calculated in order to determine whether the clumping of the hosts affected the degree of clumping of the mistletoe.

**REPRODUCTIVE DATA**

In this section certain aspects of the reproductive biology of both species were investigated, including the pollen to ovule ratios; percentage of female flowers that set seed; the distribution of seed-bearing plants in the population, as well as pollination and dispersal.

**Pollen to ovule ratios**

Pollen to ovule ratios cannot be used to test any of the hypotheses for the evolution of dioecy directly, but they do provide an indication of the relative amounts of male investment made by each species. Also, if pollen is the attractant for pollinators, then a difference between the average number of pollen grains per flower of each species would indicate from which species a pollinator could collect the most pollen in the least number of floral visits.

In order to calculate the pollen to ovule ratios it was necessary to calculate the relative numbers of male and female flowers in each population, the number of ovules per female flower and the average number of pollen grains per male flower for each species. In *Viscum capense*, the flower-bearing branches in both males and females are usually between 5 and 10 cm long. Counts were, therefore, made of the numbers of flowers per 5 cm of stem in both sexes. The average number of flowers per 5 cm of stem and the standard deviation were then calculated for each sex.

In *Viscum rotundifolium*, three flowers are presented in each cymule. The most convenient method of determining the relative numbers of male and female flowers per plant, in this case, was to sample these cymules and determine how many male and female flowers there were in each cymule.

None of the members of the family Viscaceae form ovules in their female flowers, instead, a placental mass from which the embryos develop is present (Barlow 1964). Although each flower produces only a single seed, multiple embryos may develop from one seed or dispersal unit (Johansen 1950). Since an ovule count is impossible in this family, seeds from both species were collected and were allowed to germinate in petri dishes. The number of embryos developing

from single seeds was noted and the maximum number found in each species was used in place of an ovule count for calculating the pollen to ovule ratios.

Normally, pollen counts involve dissection of anthers on a microscope slide and a count of the pollen grains is made. In *Viscum* the anthers are sessile, fused to the petals of the male flowers and consist of moist spongy tissue. The anther surfaces are covered with large pores through which the pollen grains are released (plate 1.1). Dissection of these anthers produced a mass of gelatinous tissue amongst which it was impossible to discern any pollen grains. Scanning electron microscopy of male flowers had, however, revealed that after the flowers had been put through a dehydrating alcohol series the bulk of the pollen grains appeared to have been washed out of the anthers and the anther pores appeared almost empty.

Since this seemed to be a simplest method of extracting the pollen, male flower buds which were collected just prior to opening, were dissected open and placed individually into a 1 ml volume of alcohol containing cotton blue in lactophenol (Radford et al 1974). The containers were agitated and pollen grains in suspension were later counted using a haemocytometer. Little clumping of pollen was noted so it was hoped that these counts provided an adequate measure of the number of pollen grains per flower. A total of nine squares appeared on the microscope slide surface and the pollen grains in each of these squares were counted. Six counts were made for each flower and the number of pollen grains in this volume (i.e. six fields of view) was converted to the number which would then be expected to be present in the full 1 ml volume into which each flower had been placed. Cotton blue in lactophenol stains cytoplasm and thus it was possible to determine the numbers of viable and inviable pollen grains. The average number of pollen grains per flower was therefore determined as well as the number of aborted pollen grains. Although it is likely that not all of the pollen grains were washed out of the anthers, the same method was used for both species and the results should therefore be comparable. It should be remembered however, that the results in both cases are likely to be an underestimate of the total number of pollen grains which are produced by each flower.

Using the above information, the pollen to ovule ratios for each species were calculated in the

following ways. For *Viscum capense* the average number of pollen grains per flower was multiplied by the average number of male flowers per 5 cm of stem on the male plants. This value was then multiplied by the relative number of male plants in the population. This was then divided by the product of multiplying the maximum number of embryos germinating from one seed by the average number of female flowers per 5 cm stem on the female plants and the relative number of female plants in the population. This gave the pollen to ovule ratio.

In *Viscum rotundifolium*, the average number of pollen grains per flower was multiplied by the mean number of male flowers occurring in the inflorescence cymules. This value was then divided by the value obtained by multiplying the maximum number of embryos germinating from a single seed by the mean number of female flowers which occurred in the cymules.

**Reproductive success**

A high percentage of reproductive failure in the monoecious species could be indicative of incompatibility systems or pollen clogging, which would indicate that dioecy may be advantageous under these circumstances, in terms of one of Bawa and Opler's (1975) hypotheses, by leading to greater pollination success.

For *Viscum capense*, the percentage was calculated by counting the number of fruits per 5 cm of stem at the end of the flowering season. The mean value was calculated and expressed as a percentage of the mean number of flowers per 5 cm of stem calculated above. For *Viscum rotundifolium*, the numbers of fruits produced in each cymule of 3 flowers were counted and the mean and standard deviation were determined. The mean value was then expressed as a percentage of the mean number of female flowers per cymule.

It is also interesting to compare the pollen to ovule ratios of each species with the percentage of female flowers that set seed in each species. This information can be used to compare the relative reproductive success of each species, and therefore, the success of each breeding system.

**Agamospermy**

In order to test for the presence of agamospermy, portions of the female plants of *Viscum capense* were bagged just prior to the flowering season. In *Viscum rotundifolium*, it was not possible to bag single flowers, so the central flowers of several cymules were removed and the portions of the plants from which these staminate flowers were removed, were bagged.

One of the hypotheses for the evolution of dioecy suggests that the distribution of seed-bearing plants in a dioecious population may be advantageous, since only half the population bears seed, thus making the seeds more difficult for predators to locate (Janzen 1971). In order to test whether there was a difference in the distribution of the seed-bearing plants of the two species, the average number of seed-bearing plants per tree was determined for each species. In addition, the degree of clumping of the seed-bearing plants was also determined using the method of Clark and Evans (1954). Whichever species had the highest number of seed-bearing plants per tree, or had the highest degree of clumping of its seed-bearing plants, would be the most vulnerable to seed predation. However, as mentioned in the literature review, this may be an asset in terms of attraction of dispersers.

**Pollination**

Information regarding the pollination of different species can give an insight into the extent of gene flow within and between populations, and thus, can be important in estimating the extent of inbreeding and outbreeding occurring in populations. In this way, pollination can be used to make inferences about the evolution of dioecy. Since observations of the study species had shown that the flowers differed only in size, it was assumed that the pollination syndrome of each species was likely to be similar. The aim of this section of the study then, was primarily to determine whether or not the two species of *Viscum* had different pollinators and what the consequences of these pollinators would be in terms of gene flow.

The extensive laboratory work required for the genetic section of the study limited the amount of time which could be spent in the field, so that extensive pollination biology studies were impractical. General descriptive data were therefore used to determine whether any marked differences in pollination existed between the species.

For *Viscum capense*, an area was selected in which three host trees occurred, each tree containing a number of male and female plants. Observations of pollination were made throughout the flowering seasons whilst other data were being collected. Approximately midway through the flowering season of this species (late August), a day was set aside and spent observing pollinators from 6am to 6pm. Several floral visitors were trapped using an insect net and were later identified to the level of family. The behavior of the visitors to both species was observed in order to determine whether gene flow was affected in either species by the type of pollinator. The presence of several host trees in the area made it possible to determine how long pollinators spent within a single host tree before moving to another, as well as how long they

observed in order to determine whether gene flow was affected in either species by the type of pollinator. The presence of several host trees in the area made it possible to determine how long pollinators spent within a single host tree before moving to another, as well as how long they spent visiting the flowers of a single plant. The time of day at which pollinator activity was highest was also noted, as well as whether male and female flowers were visited at different times of day. An attempt was also made to determine, by observation, which floral resources were being utilised by the pollinators. Similar detailed observations were undertaken for *Viscum rotundifolium* the following year, towards the end of January, approximately midway through its' flowering season.

**Dispersal**

*Viscum* seeds are dispersed by birds, and as with pollination, a limited amount of field work could be undertaken in terms of bird watching. There is, however, literature available on the types of birds responsible for the dispersal of mistletoe seeds and the manner in which the birds handle the fruits and seeds (Godschalk 1986). In a study undertaken at Loskop Dam in the Transvaal, it has been shown that different species of birds handle fruits in different ways, but specific behavioural patterns are adhered to within a species (Godschalk 1986). Thus the types of birds present in an area will affect the dispersal of fruits.

Lists of the bird species which occur at Makapan's gat had already been compiled by an ornithologist for April of 1986, 1987 and 1988 (Weissenbacher unpublished data). Some of the frugivores on these lists corresponded to species involved in mistletoe dispersal at Loskop Dam and the role of these particular birds in the dispersal of mistletoe seeds, as recorded by Godschalk (1986), is documented in the results. Roberts' Birds of Southern Africa (Maclean 1984) was consulted to determine which of the birds on the lists obtained from Weissenbacher were frugivores, and these too are recorded in the results section. The breeding seasons of these birds were also noted, since they would remain in one locality for the duration of this period and this would affect dispersal. The seasons during which the fruits of the two species were available have also been noted.

The data obtained for the degree of clumping of the two species was also used for making some inferences about dispersal in these species. In addition to this, another test was devised to determine whether dispersal of seed was random or whether seeds were preferentially dispersed within trees with existing seed-bearing females. The average number of plants per tree was

obtained for host trees which contained only male plants and for those trees which contained at least one seed-bearing plant. It was assumed that if seed was preferentially dispersed within trees containing existing seed-bearing plants, then trees containing seed-bearing plants should have a significantly higher number of plants per tree than those hosts which contained no females.

## GENETIC DATA

This section of the study set out to address the hypotheses which invoke selection for outcrossing as the driving force for the evolution of dioecy. Whether one believes the advantage of outcrossing to lie in its prevention of inbreeding depression, or the increase in the number of heterozygous individuals one would expect to appear in the population, the underlying assumption is that the advantages would only be realized if there was an increase in heterozygosity as a result of outcrossing. The validity of these hypotheses for the evolution of dioecy in *Viscum* can, therefore, be tested by determining whether or not the dioecious population has a higher level of heterozygosity than the monoecious population. If it does, it would indicate that selection for dioecy may have operated through selection for outcrossing or increased heterozygosity. If no difference in heterozygosity between dioecious and monoecious plants could be found, there would be fairly strong evidence for rejecting the above hypotheses in the case of *Viscum*.

### Starch gel electrophoresis

The method chosen to determine the levels of heterozygosity in the populations was starch gel electrophoresis of enzymes. This technique involves the separation of the different molecular forms of an enzyme, or protein, by loading crude extracts of plant tissue on to starch gels and applying an electric field. The enzyme variants migrate at different rates due to their having different electrostatic charges, sizes and configurations (Gottlieb 1977). The relative positions of the enzyme variants on the gels are visualised by staining reactions based on the catalytic activities of the enzymes, so that particular enzymes can be distinguished from among the many that may be present in the original crude extract (Gottlieb 1977).

A single reaction may be catalysed by different molecular forms of an enzyme, which are called isozymes if their polypeptide constituents are coded for by more than one gene locus. Different molecular forms of an enzyme whose polypeptides are coded for by different alleles at the same locus are called allozymes (Gottlieb 1977). Allozymes and isozymes are therefore direct products of the genes since they are coded for by the nucleotide sequence of the gene locus, and since

they have different molecular structures, this usually causes changes in electrophoretic mobility. Electrophoresis thus makes it possible to determine genotypes at specific loci (Gottlieb 1977).

The number of bands which appear on a gel does not only depend on the number of loci and alleles coding for a particular enzyme, but also on the subunit structure of the particular enzyme (in other words, how many polypeptides the enzyme consists of) (Gottlieb 1977). For example, in the case of a single locus which is heterozygous coding for an enzyme consisting of a single polypeptide, two allozyme bands would be present. If the enzyme were dimeric (composed of two polypeptides), three bands would appear and if it were tetrameric, five bands would be present. This situation is complicated further when more than one locus is involved (Gottlieb 1977). Prior knowledge of the subunit structure of the enzymes one uses in a study obviously aids in interpretation of banding patterns.

This form of electrophoresis is a useful genetic tool since it has the following advantages (Brown & Weir 1983):

(a) Apart from a few exceptions, the enzyme bands show codominant inheritance and segregate as single Mendelian factors;

(b) Enzyme specificity allows attribution of alleles to loci and the comparability of loci in different populations or species ;

(c) Each allelic difference is detected as a mobility difference which is independent of the functional role, or the overall level of variation of the enzyme in question;

(d) The loci sampled are frequently determined by the tissue expression, suitable extraction and availability of assays for zymograms so that the loci are effectively sampled at random.

There are also some disadvantages of this technique listed by Brown and Weir (1983) and these include:

(a) The possible occurrence of post-translational modification of electrophoretic mobility;

(b) Duplication of genetic material, as in polyploids, can often lead to the inability to ascribe a particular variant to a single locus;

(c) Not all base substitutions result in amino acid replacements which alter the net charge on

the protein to the extent that the change will be detected by electrophoresis.

Electrophoresis of isozymes has been used widely in systematics to determine phylogenies as well as in the determination of genetic identity and divergence between species, genera and families (Crawford 1985 and Gottlieb 1977). The effect of certain life history characteristics on population genetic structure has also been investigated using this technique (Hamrick, Linhart & Mitton 1979). In a similar fashion, Brown (1979) investigated the relationship between breeding systems and population genetic structure. Although the data obtained in this study could be used to investigate some of the above areas of research, for the purposes of the present study, the levels of genetic heterozygosity in each population were all that was required.

**Application of the technique**

The actual procedure of electrophoresis has been described fully in a number of publications (Cardy et al 1983, Cheliak & Pitel 1984 and Shields et al 1983) and will not be discussed further here. Since no prior genetic analyses of *Viscum* using electropheresis had been undertaken, it was not known which extraction buffers, running buffers and gel buffers would be most suitable for resolving allozymes of these species. For this reason, preliminary tests of extraction procedures, extraction buffers and different combinations of gel and electrode buffers, had first to be carried out. Several plants of both species were collected and ground in the following extraction buffers, the recipes for which are included in the appendix.

Phosphate grinding buffer - PVP solution (Soltis et al 1983)

Tris - HCl grinding buffer - PVP solution (Soltis et al 1983)

Tris - maleate grinding buffer - PVP solution (Soltis et al 1983)

Cheliak & Pitel grinding buffer - PVP solution (Cheliak & Pitel 1984).

The buffer which gave the best resolution of bands and the greatest amount of activity over a number of different running and gel buffers was used for the remainder of the study. Numerous enzymes having different metabolic functions, were stained for on several of the running and gel buffer systems. When staining was completed, gels were fixed in a volume-to-volume ratio of 5:5:1 glycerol:water:acetic acid (Cardy et al 1983). After soaking in fixer for 24 hours the gels were allowed to air-dry and were then stored indefinitely in plastic wrap. Recipes for all the

extraction buffers and the tray and gel buffers are included in the appendix. Enzyme staining schedules were obtained from Soltis et al (1983).

**Levels of heterozygosity**

In this study, electrophoresis was used to determine the levels of heterozygosity in the monoecious and dioecious populations by noting the number of bands occurring on the gels for each individual of the two populations at Makapansgat for a number of different loci (Gottlieb 1977). By consulting published subunit structure information for each enzyme, it was possible to determine whether the banding patterns for each plant represented a homozygous or a heterozygous state at each locus. The percentage of heterozygous individuals for each locus was noted and from this the average populational levels of heterozygosity were calculated by summing the locus-by-locus results and obtaining the mean.

In populations which are large and in which mating occurs at random, it is possible to predict the proportion of heterozygotes one would expect in the population from data on the frequency of alleles in the population using the Hardy-Weinberg formula (Nei 1987). The allelic frequencies can be determined from the gels for each locus (Crawford 1985 and Gottlieb 1977) and since the level of heterozygosity has already been calculated for each locus, it is possible to compare the observed levels of heterozygosity with the expected levels from the Hardy-Weinberg formula. When two alleles are present in the population, the frequency of the alleles is designated as p and q respectively. The proportion of homozygotes of each allele can then be calculated by $p^2$ and $q^2$ respectively. The proportion of heterozygotes in the population can be calculated by 2pq. Wright's F statistic is a measure of the departure from Hardy-Weinberg equilibrium at each locus and is determined using the observed levels of heterozygosity and those expected from Hardy-Weinberg calculations (Wright 1965). F statistics are calculated in the following way:

$$F = 1 - \frac{\text{Observed frequency of heterozygotes}}{\text{Expected frequency of heterozygotes}}$$

An F value of 0 means that the population is in Hardy-Weinberg equilibrium. A negative number indicates an excess of heterozygotes, with a value of -1 indicating that all individuals are heterozygous. A positive value indicates an excess of homozygotes, with a value of 1 indicating that all individuals are homozygous.

Another method of calculating deviations from Hardy-Weinberg proportions, Levene's method, which takes into account population size, was also calculated for each locus (Nei 1987).

Loci were named after the enzymes that they coded for. Whenever more than one locus was found to code for variants of the same enzyme, the loci where also given numbers. Loci where numbered in ascending order according to the distance their subunits migrated, with the locus whose subunits migrated the greatest distance being given the number 1.

Bands on the gels which represented alleles were also named according to the distance their subunits migrated. At each locus, the subunit which had travelled the greatest distance through the gel was named A. Other subunits at the same locus where then named B, C or D, depending on the distance they migrated.

**Distribution of alleles**

Since additional populations from other sites were used in the genetic analyses, novel alleles occurring in some of the populations of species, and not in the others, were noted to determine whether any intraspecific genetic differences occurred between populations. In this way the effects of founders in these species could be examined, as well as the extent to which migrations had influenced the genetics.

Finally, since all the host trees at Makapansgat and the plants in them were labelled, it was known which genotypes corresponded with which plants in the population, as well as the relative positions of those plants. Thus, it was possible to determine for the two populations at Makapansgat, whether any unique gene combinations occurred in single trees only, or whether all genotypes were distributed randomly throughout the populations.

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