RAPD variation within and between natural populations of morama [*Tylosema esculentum* (Burchell) Schreiber] in southern Africa

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RAPD markers were used to investigate genetic variation in natural populations of morama [*Tylosema esculentum* (Burchell) Schreiber]. This species is a wild, perennial, outbreeding legume indigenous to the Kalahari Desert region of southern Africa. Morama seed has a high protein content and has traditionally been an important wild source of food for the people of this region. It is considered to have great potential for development as a new crop for the semi-arid tropics. Investigation of genetic diversity is a prerequisite for the genetic improvement of any species and this is the first such investigation in morama. Considerable RAPD variability was detected in each of the three morama populations sampled. Most of this variation (85%) occured within, rather than between, populations in agreement with other studies involving outbreeding perennial species. This suggests that sufficient genetic variation for breeding purposes may be found by sampling 30–40 plants from only one or two populations. There were significant differences in the frequency of RAPD loci between populations but no population-specific RAPD markers were found. Although there was no clear evidence of population differentiation between the morama populations, more intensive sampling may reveal details of within-population genetic structure in these populations. This analysis confirms the usefulness of RAPD markers for investigation of genetic variation in natural populations and the technique is fast and provides numerous polymorphic markers.

Keywords: Population genetic variation, RAPDs, morama, outbreeding perennial legume.

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Introduction

The morama plant [*Tylosema esculentum* (Burchell) Schreiber] is a drought-tolerant legume (subfamily Caesalpinoideae) native to the Kalahari Desert region of southern Africa. Morama is a long-lived, perennial species which regenerates annually from a large underground tuber. The above-ground vegetation consists of numerous prostrate vines which can reach up to 6 m in length. Although morama has been shown to be self-compatible (De Frey 1990), a distylic floral morphology means that it is predominantly outbreeding. The seeds from morama have traditionally been an important source of protein for the inhabitants of the Kalahari region. The plant has never been cultivated by these people, but is harvested from extensive populations which are scattered throughout its natural range.

Protein contents ranging from 30–35% and oil contents from 35–42% have been recorded in deshelled morama seed (Wehmeyer *et al.* 1969; Bower *et al.* 1988). This compares very favorably with the nutritional value of many existing legume crops and as a consequence, a USA National Academy of Sciences (1979) report has described morama as a plant of considerable potential for semi-arid agriculture. Its potential has also been highlighted by Keegan and van Staden (1981), Miller and Powell (1981) and Bousquet (1982). Powell (1987) has demonstrated that the morama plant can be successfully cultivated during trials in the USA. More recently, research efforts have been initiated in Australia which will evaluate and select superior plants for cultivation.

To date, however, there has been no investigation of either the amount of genetic diversity present, or of the distribution of variability within and between populations of morama. This information is a prerequisite for the genetic improvement of any plant species. Given that the natural populations of morama are under pressure from both grazing and human exploitation of its seed, knowledge of the genetic structure of these populations is also important for developing a strategy for conserving the remaining wild germplasm.

Recently, DNA-based procedures have replaced the use of allozyme analysis in many investigations of genetic diversity. Of these new procedures, the randomly amplified polymorphic DNA (RAPD) technique has been preferred over both restiction fragment length polymorphisms (RFLP) and the use of minisatellite DNA sequences in population-based studies because it is less laborious and allows a more rapid assesment of genetic variation (Russell *et al.* 1993). It has now been used successfully to measure diversity among wild populations of a number of species including *Gliricidia* spp. (Chalmers *et al.* 1992), *Buchloe dactyloides* (Huff *et al.* 1993), *Elacis guineensis* (Shah *et al.* 1994) and mahogany (Meliaceae) species (Chalmers *et al.* 1994). The present study has used RAPD markers to estimate the levels of genetic variation in three geographically separated natural populations of morama.

Materials and Methods

Sample collection

Fresh young leaves were collected from plants selected at random from sites (approximately 1 ha in area) in each of three morama populations in Botswana (Figure 1). These are separated by distances of approximately 600–800 km but occur in similar dry savannah environments (with annual rainfall ranging from 350 to 600 mm) and have similar plant densities (300–400 plants ha^{-1}). Sixteen plants sampled from each of the populations were used in the analysis.

DNA extraction

DNA was extracted from approximately 300 mg of the frozen leaf samples. The leaf material was ground to a fine powder in liquid nitrogen in a mortar and pestle. It was then placed in a microcentrifuge tube with 650 µl of extraction buffer, ground again gently with a glass rod and incubated at 65°C for 30 min with an occasional inversion of the tubes.

The extraction buffer was composed of 2.0% CTAB, 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 1.0% PVP (mw 40 000) and 0.1% mono-thioglycerol. Following incubation, 650 µl of chloroform:iso-amyl alcohol (24:1) was added and mixed thor-

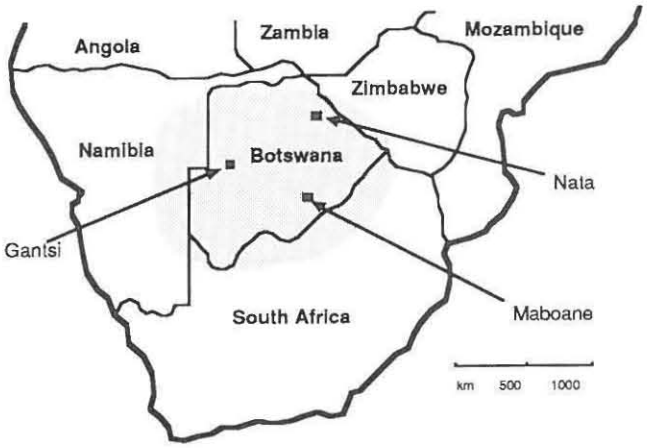

Figure 1 Approximate natural range of morama and locations of sampled populations.

oughly. The mixture was then centrifuged at 13 000 r.p.m. for 30 sec and the supernatant transferred to a new tube. A $^1/_{10}$ volume of CTAB (10% CTAB, 0.7 M NaCl) was added at 65°C, gently shaken by hand and then re-extracted with one volume of chloroform: iso-amyl alcohol. This was again centrifuged at 13 000 r.p.m. for 30 sec and the supernatant transferred to a new tube. Then 50 µl of 2 M Na acetate (pH 5) together with two volumes of cold 100% ethanol were added and mixed thoroughly before being stored in the freezer for 30 min. The mixture was centrifuged at 13 000 r.p.m. for 15 min, the supernatant was discarded and the remaining pellet was washed twice in 70% ethanol before being dried in a speedivac (Savant brand) for approximately 10 min. The pellet was dissolved in 200 µl of TE buffer (1 mM Tris-HCl pH 8, 0.1 mM EDTA) and stored at 4°C.

DNA amplification

A set of twenty 10mer primers of 60–70% C+G content were obtained from Operon Technologies (kit A). One of the 20 primers was randomly selected for each amplification run until reproducible and scoreable banding patterns were obtained from at least 10 different primers.

Two microlitres of DNA from each sample (containing approximately 40 ng of template DNA as determined with a spectrophotometer) was added to the side of a microtube and kept on ice. A master mix containing 660 µl sterile water, 120 µl of 10 × reaction buffer (Boehringer), 240 µl of a stock solution with 0.5 mM each of dATP, dCTP, dGTP and gTTP (Boehringer), 48 µl of a 5-µm solution of the primer, 24 µl of 25 mM $MgCl_2$ (to bring the concentration of $MgCl_2$ in the reaction mix to 2 mM) and 12 µl of Taq polymerase (Boehringer) was prepared and briefly vortexed to ensure good mixing. Of this mix, 23 µl was added to each microtube (= a total of 25 µl), making sure that it mixed with the drop of DNA on the way down, and it was briefly spun down in a centrifuge to ensure that all of the mixture was at the bottom of the tube. A drop of mineral oil was then placed on top of the mixture and the tubes were quickly transferred to the thermal cycler. Another drop of mineral oil had previously been added to each well in the cycler which had been preheated to 80°C. The cycling programme began with an initial 5 min at 94°C followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, and then 72°C for 2 min. The PCR samples were then stored at 4°C until evaluated by electrophoresis in a 1.25% agarose gel containing 5 µg ml^{-1} ethidium bromide. The reaction mixture (20 µl) was mixed with 5 µl of gel-loading buffer and 20 µl of this was applied to each well in the gel. A 100-kb DNA ladder sample was added to the outside well in each gel. The gels were run at a voltage of 5 V cm^{-1} for about 2 h. They were then visualized immediately under UV light (x = 204 nm) and photographed with a MP-4 polaroid system.

With all the primers used, the PCR reaction failed in one or two individuals and these were subsequently repeated. At the same time, three other samples from each population were also repeated to check for reproducibilty. Blanks were also run to check for any contaminating DNA. Bands were scored only if they appeared within a 'window' of more intensely stained bands of intermediate molecular weight (usually between 500 and 1 600 base pairs). Only those bands within this window, which were relatively clearly present or absent, were scored. Any scoreable bands which were not reproduced in the selected repeats were eliminated from the analysis.

Data analysis

Each RAPD band was scored as a binary character, 0 for absence and 1 for its presence. The genetic diversity within each morama population was measured using the Shannon information index (King & Schaal 1989) which is calculated as:

$$H = \sum_{i=1}^{k} p_i \log_e p_i$$

where p_i is the frequency of the ith band among all individuals within a population. H was averaged over all bands including those which were monomorphic (i.e. $p_i = 1$) to give the average per-locus diversity within populations (H_W). The average population diversity (H_T) was then derived from the three separate measures of H_W. The genetic diversity of the species (H_S) was calculated using band frequencies for all individuals from the three populations. The proportion of genetic diversity within populations is given by H_T/H_S and that due to variation between populations by $(H_S-H_T)/H_S$.

A pair-wise similarity matrix between all individuals was generated using the matching coefficient (Gordon 1981):

$$s_{ij} = \frac{a+d}{a+b+c+d}$$

where a is the number of bands that are present in both i and j individuals, b is the frequency of bands that are present in i but not in j, c is the frequency of bands that are present in j but not in i and d denotes the frequency of bands that are absent from both individuals. It should be noted that this matching coefficient differs from the more commonly used coefficients of both Nei and Li (1979) and Jaccard (1901) by including the shared absence of bands in measuring the degree of similarity between two individuals. It is considered that at a population level this gives a more accurate view of the relationship between individuals from within that population. The extensive similarity matrix generated was then reduced to population means and subjected to hierarchical cluster analysis using both the single-link and average fusion strategies (Gordon 1981). The similarity matrix was also used as an input into principal coordinate analysis (Gower 1966). These analyses were performed with the Genstat 5 statistical package.

Results

The RAPD profile

Thirteen primers were tested. All of them produced polymorphic markers with a size range of 400 to 2 000 base pairs, but primers A7 and A8 were considered too difficult to score reliably and were eliminated from the analysis. The remaining 11 primers produced 86 scoreable bands (Table 1) of which 73 (85%) were polymorphic. The total number of bands scored per primer ranged from 4 (A9) to 12 (A20 and A11). The primers with the greatest number of polymorphic bands were A18 with 11 (92% of bands scored), and A11 with 12 (100% of bands scored). All the individual plants analysed had unique genotypes, that is, no two plants had the same RAPD profile. There were no unique bands which were fixed in one population and absent in another,

Table 1 Total number of loci examined, number of polymorphic loci and loci that occur at significantly different frequencies between the morama populations for the 11 primers scored

Primer	Total no. of loci	No. of polymorphic loci	No. of loci showing significant differences (% of polymorphic loci) Nata vs. Maboane	Nata vs. Gantsi	Maboane vs. Gantsi
A20	12	9	6	5	9
A19	7	6	2	1	3
A18	11	11	6	4	5
A14	6	5	1	3	3
A13	7	3	2	1	1
A11	11	11	3	7	8
A09	4	3	1	0	0
A05	7	6	4	1	3
A03	6	5	0	1	1
A02	5	4	0	0	0
A01	10	10	5	3	1
Total	86	73	30 (41.1)	26 (35.6)	34 (46.6)

but regression analysis revealed significant differences ($P = 0.05$) in band frequency between the three populations for a number of loci (Table 1). The greatest number of significantly different loci (46.6%) occurred between the Maboane and Gantsi populations and the least number of differences (36.5%) were observed between the Nata and Gantsi populations.

Within- and between-population diversity

The average population diversity using the Shannon information measure for the three morama populations was 0.1752 and ranged from 0.1663 for Maboane to 0.1898 for the Gantsi population (Table 2). This suggests that Gantsi is the most diverse morama population, but the ranking of populations in terms of highest to lowest diversity changed with the primer used. Primer A1 revealed the highest individual level of diversity of 0.2646 in the Gantsi population, but the greatest estimate of average population diversity was revealed by primer A2 (0.2192). The lowest amount of diversity for an individual population (0.0327 for Nata) and for average population diversity (0.0783) was revealed by primer A13.

Most of the diversity (84.6%) in morama populations occurs within rather than between (15.4%) populations, however, this estimate varies between primers. For example, primer A20 detected up to 31.3% of variation between populations while primer A2 indicated that as little as 3.2% of the total variation was due to variation between populations. Together with the fact that primer A2 produced the greatest estimate for average population diversity, this result is consistent with the observations that primer A2 failed to yield any significant differences in band frequencies between any of the population comparisons (Table 1). In other words, although primer A2 is relatively polymorphic, it does not differentiate populations. Primer A20, on the other hand, produced the greatest number of significant differences,

Table 2 Partitioning of the genetic diversity within and between populations of morama for 11 random primers

Primer	Genetic diversity within populations (H_W) Nata	Gantsi	Maboane	Average within-popn diversity (H_T)	Total genetic diversity (H_S)	% diversity within popns.	% diversity between popns
A20	0.1480	0.1602	0.1228	0.1437	0.2093	68.7	31.3
A19	0.2088	0.1677	0.1512	0.1759	0.1986	88.6	11.4
A18	0.1870	0.2410	0.1723	0.2001	0.2371	84.4	15.6
A14	0.1864	0.1652	0.1760	0.1759	0.2027	86.8	13.2
A13	0.0327	0.1035	0.0987	0.0783	0.0998	78.5	21.5
A11	0.1245	0.1540	0.1925	0.1570	0.2085	75.3	24.7
A09	0.1850	0.1710	0.1800	0.1787	0.1902	93.9	6.1
A05	0.1748	0.2345	0.1372	0.1822	0.2037	89.4	10.6
A03	0.1847	0.2111	0.1795	0.1918	0.2021	94.9	5.1
A02	0.2223	0.1852	0.2502	0.2192	0.2264	96.8	3.2
A01	0.2373	0.2646	0.2230	0.2116	0.2633	80.4	19.6
Total	0.1694	0.1898	0.1663	0.1663	0.2072	84.6	15.4

but gave a low estimate (0.1437) of genetic diversity within the populations. Consequently, it may be a more useful primer than the others for differentiating morama populations.

A reduced matrix showing the mean similarity within and between each population is given in Table 3. Individuals in the Gantsi population displayed the least degree of similarity to each other (73.0), supporting the previous estimate of that population's greater diversity using the Shannon measure. However there was very little difference in the mean similarities of individuals in each of the three populations. The mean similarity in the Nata population was 75.1, while plants in the Maboane population displayed a mean similarity to each other of 76.1. There were also no significant differences in mean similarities between populations when compared to mean similarities within the populations. Plants in the Maboane population were statistically as

Table 3 Reduced RAPD similarity matrix based on the mean of all individual pair-wise comparisons for each population. The mean similarity within populations is represented by the leading diagonal

	Nata	Gantsi	Maboane
Nata	75.2		
	SE = 0.40		
	SD = 4.35		
Gantsi	70.8	73.0	
	SE = 0.28	SE = 0.48	
	SD = 4.47	SD = 5.28	
Maboane	71.1	69.1	76.1
	SE = 0.27	SE = 0.35	SE = 0.37
	SD = 4.39	SD = 5.64	SD = 4.05

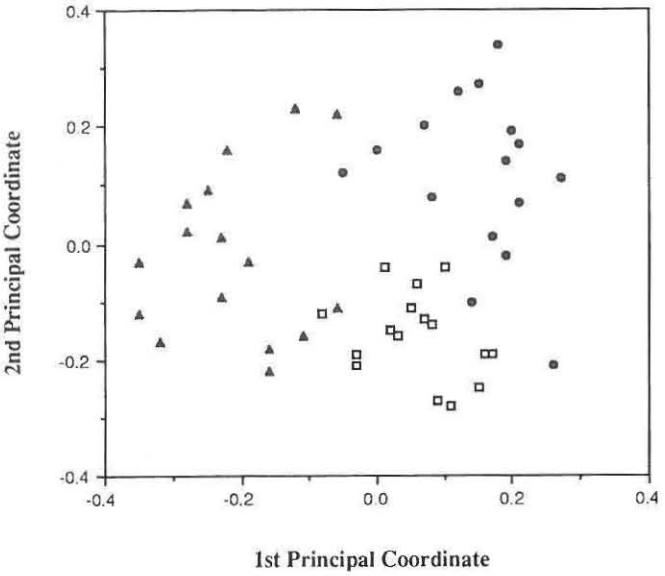

Figure 2 Principal coordinates analysis of the three morama populations: ●, Maboane; ▲, Gantsi; □, Nata.

similar to plants from the Nata population (mean similarity of 71.1) and also to the Gantsi population (mean similarity of 69.1) as they were to each other. Cluster analysis of the similarity matrix using both single and average linkage failed to produce any obvious separation of individuals into their respective geographic groups.

However, the relationship between the three morama populations can also be represented by the principal coordinate analysis (Figure 2). With the exception of a couple of outliers, the ordination separated individuals into the three population groups. The Maboane and Nata populations appeared to show the greatest differentiation which is in conflict with the mean similarity data given in Table 3. However it should be noted that the two principal coordinates in Figure 2 represent only 19% of the total variation and do not fully represent the relationship between the populations. Therefore, the relatively neat separation of the populations observed in Figure 2 is not reliable evidence of genetic differentiation between the three populations.

Discussion

This study again confirms the usefulness of RAPDs in providing a quick method of investigating genetic variation in plant populations. The average number of bands scored per primer (7.8) using morama DNA was similar to that reported for cocoa (*Theobroma cacuo* L.) by Russell *et al.* (1993) and coconut (*Cocos nucifera* L.) by Ashburner (1994), however, it is less than that reported in a number of other investigations. For example, Chalmers *et al.* (1994) detected 13 per primer in mahogany species (Meliaceae) while Huff *et al.* (1993) scored as many as 18.6 in buffalo grass (*Buchloe dactyloides*). This does not necessarily indicate that RAPD markers are less numerous in morama and therefore potentially less useful for the analysis of its genetic variation. Considering the fact that the selection of bands for scoring is somewhat subjective and that there are many different primers available, it would seem that for most species, given sufficient effort and careful selection of primers, a similarly large number of RAPD bands could be identified and used in the analysis of genetic variation.

The various elements of the RAPD analysis conducted in this study suggest a considerable amount of genetic variation exists in morama and that most of this variation occurs within, rather than between, populations. Since morama is a widely distributed, perennial, outcrossing species, this result fits the general pattern of genetic variation, which Hamrick and Godt (1990) suggest is typical of such species. However their conclusion has been based on isozyme analysis. Whether or not the accumulated information from RAPD anayses reveals a similar pattern remains to be seen. Chalmers *et al.* (1992) suggest that because isozymes represent only the coding regions of the genome, isozyme data may not be directly comparable with RAPD data. Unfortunately because of the fact that there are still relatively few studies on wild populations which have used RAPDs and also the slightly different approaches used in these few RAPD analyses, meaningful comparisons of the level of variation in morama with other species of similar life history are difficult to make. However the high percentage of polymorphic loci (85%) is one indication of a relatively high level of genetic diversity in morama populations. It compares with the 87% observed by Lui and Furnier (1993) in big-tooth aspen (*Populus grandidentata*) and 75% in cocoa (Russell *et al.* 1993). Ashburner (1994) found only 67% of all loci were polymorphic in coconut and Chalmers *et al.* (1992) only 65% in *Gliricidia sepium*. All of these are perennial, outcrossing species.

The Shannon diversity index for total diversity that was determined in this analysis (H_t = 0.207) is less than in other studies which have used this as a measure of genetic diversity. For example, Ashburner (1994) found H_t = 0.345 in coconuts and Shah *et al.* (1994) produced indices ranging from 0.24 to 0.46 in populations of oil palm. However the lower measure observed in morama may be explained by the fact that in both these studies monomorphic RAPD bands were excluded from the analysis. It is our view that the inclusion of monomorphic RAPD bands provides a more realistic picture of the level of genetic diversity in plant populations. Many analyses (such as Chalmers *et al.* 1992; Russell *et al.* 1993; Shah *et al.* 1994) have also used $\log_2$ rather than $\log_e$ in the calculation of the Shannon index. This has the effect of increasing the measure by a factor of 1.44. Both Chalmers *et al.* (1992) and Russell *et al.* (1993) also complicate comparisons in measures of diversity between different investigations by apparently using only the sum of $P_i \log_e p_i$ (described earlier in methods section) to derive the diversity measure and not the average over all loci. Dividing their reported Shannon diversity indices (2.96 and 2.55) by the apparent number of loci they examined and converting $\log_2$ to $\log_e$ produced figures of H_t = 0.209 (Chalmers *et al.* 1992) and 0.217 (Russell *et al.* 1993) for these two species, which compare more favourably with the measure of 0.207 observed in the present study for morama.

The fact that despite geographic separation, there apparently has been very little differentiation between the morama populations is not unexpected, given the relatively uniformity of the dry savannah environments in which they occur. A single-locus estimate of t = 0.77 (Monaghan 1995) supports the proposition that morama is predominantly outcrossing. Morama is pollinated by a number of insect species and occurs in large extensive populations (pers. obs.). Therefore, the effective population size in morama may be sufficiently large so as to reduce the influence of genetic drift. Gene flow occuring as a result of seed movements, either by grazing animals or people moving from region to region with harvested seed, may have further retarded the process of differentiation between morama populations.

Little is known of the origins of morama. Various anthropological studies (e.g. Schapera 1937) have indicated that the Bantu-speaking people of southern Africa originated from east Africa and that their gradual migration southwards may have pushed indigenous hunter–gatherer groups into the drier Kalahari regions. It is interesting to note that although morama is restricted to southern Africa, the other three species in the *Tylosema*

genus are distributed throughout eastern Africa from the Sudan southwards. According to Watt and Breyer-Brandwijk (1962), *T. fassoglensis* has also traditionally been collected for food by people in Tanzania, so the distribution of the genus may be associated with this southern migration. The fact that morama occurs only in southern Africa may be a result of later differentiation. The ongoing migration of people into the Kalahari culminated in the 19th century as many of the Bantu people moved westward to escape the expanding Zulu empire. Therefore it is possible that the widespread, but patchy, distribution of morama and the lack of genetic differentiation between morama populations in Botswana may be linked to the historical movements of traditional users of the plant. Isozyme banding patterns indicative of tetrasomic inheritance provide some evidence that morama may be an autotetraploid (Monaghan 1995). Since polyploids eventually tend to revert to a diploid level of gene expression (Ferris 1977), the extent to which subsequent diploidization of the genome has occurred in morama would shed some light on whether or not it is of relatively recent origin as a species. If morama is an autotetraploid, this would be another factor contributing towards the relatively high level of genetic diversity and minimal differentiation observed in this study. This is because of the increased heterozygosity associated with autotetraploidy, and the fact that the process whereby alleles become fixed in a population due to random drift and so produce differentiation between populations will be slower, in tetraploid, compared with diploid species.

The fact that 85% of all the molecular variation observed in the morama populations sampled appeared to occur within a population suggests that sampling a larger number of plants from one or two populations rather than smaller collections from many different sites should provide a sufficiently diverse germplasm base on which to develop a breeding programme for its improvement. According to Marshall and Brown (1975), 50–100 plants should be sampled from a site. Given the high level of diversity present, it is suggested that 30–40 would probably be sufficient in the initial stages of a genetic improvement programme for morama. It must be remembered, however, that this is only a preliminary study and the variation which has been measured is largely non-adaptive. More extensive investigation of both morphological characteristics and various enzyme systems which may be found to have some adaptive function, could reveal differences between populations (and individual plants) with more practical relevance for a breeding programme. Therefore, ongoing sampling in morama populations is recomended.

From the point of view of conserving morama germplasm *in situ*, the results of the present study appear to justify conserving only one or two populations for future use. Again, however, further information on breeding systems, the importance of gene flow between existing populations and the level of natural selection pressure, all of which will affect the maintenance of variation in the population and therefore its availability for future use, are needed before it can be accepted that this is a satisfactory conservation strategy.

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