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Conservation of *Acacia tortilis* subsp. *raddiana* Populations in Southern Sinai, Egypt.
II- Mating System Analysis

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A CACIA TORTILIS subsp. *raddiana* is a native species of extremely xeric habitats with poor soils in the Middle East and Africa and have experienced decline in population size and number and connectivity due to unmanaged human activities. It's restoration in natural habitats as well as its growth in commercial plantations will be improved by knowledge of its natural history and by applied breeding programs. This manuscript presents an analysis of outcrossing and inbreeding rates in *A. tortilis* subsp. *raddiana* for potential use in establishing an effective restoration and conservation strategy for natural populations from Southern Sinai, Egypt. Open-pollinated seeds from 127 families representing twelve populations were used. Seven polymorphic allozyme loci were used to estimate population outcrossing rates, levels of biparental inbreeding, and the effective number of pollen donors per maternal plant. Mean outcrossing rate (t_m) over all populations was 1.106 ± 0.080. In all populations the effective number of pollen donors per maternal tree was high indicating low correlated mating within individual trees. Biparental inbreeding was not significantly higher than zero in any population. *A.tortilis* could be effectively conserved by maintaining a few populations of sufficiently large size to reduce the loss of genetic diversity and to maintain pollinator communities to facilitate outcrossing and gene flow between populations. Restoration efforts should include many genetically unrelated individuals to prevent increased inbreeding.

Keywords: *Acacia tortilis*, Allozymes, Sinai, Conservation genetics, Mating system, Outcrossing.

Any long-term plant conservation program should conserve the genetic variation present in a species and minimize processes that reduce this variation (*e.g.* Holsinger and Gottlieb 1991). The best way to capture most of the adaptive variation present is to conserve many large populations over the ecological and geographical range of the species (Schemske *et al.* 1994). Often, however, such populations don't exist, especially in arid ecosystems, and even if they do, it is rarely possible to conserve all the populations that are desired due to economic and other factors involved in the decision making process. Thus, populations and areas are selected based on their contribution to the development of an effective

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reserve network. Systematic rather than opportunistic selection of populations and areas for *in situ* protection should be based on an understanding of how genetic diversity is structured and on predictions of future changes in that diversity (Pressey *et al.* 1993). Knowledge of mating systems aids in this understanding because of its role in structuring genetic diversity, transmitting diversity across generations, and determining rates of diversity loss (Brown 1989; Hamrick *et al.* 1979; Ritland 1989). Knowledge of current mating patterns is especially important for highly fragmented populations and/or those with greatly reduced effective population sizes.

Many species of extremely xeric habitats have experienced human-mediated reductions in population size and connectivity due to overgrazing by domestic livestock. As a result, species of considerable ecological and commercial value are now facing local and rangewide extinction. This scenario is typical of desert tree species such as *Acacia* whose characteristics make them valuable as forage for wildlife and the livestock of indigenous people. Because of their potential value for agroforestry and environmental protection many essentially wild *Acacia* species are included in international germplasm collections and are considered candidates for genetic improvement programs. Knowledge of their natural mating systems is essential for developing sampling schemes and breeding strategies that ensures the preservation of adequate levels of genetic diversity for restoration and genetic improvement programs.

Although *Acacia* is an important genus, relatively little is known about its reproductive biology. It is known that insects, especially bees, are the main pollinators (Bernhardt *et al.* 1984), but birds may also be important (Knox *et al.* 1985; New 1984). Philp and Sherry (1946) carried out pioneering studies of the breeding system of two Australian *Acacia* species and established that *A. decurrens* and *A. mearnsii* are only partially self-compatible. Several other acacias were reported as outbreeding and/or self-incompatible; *e.g A. senegal* (Tandon and Shivanna 2001) and *A. simuate* (Raju and Rao 2002). In addition to self-incompatibility, dichogamous flowering is thought to promote outcrossing in many *Acacia* species.

Acacia pollen grains are grouped into polyads with 4-16 pollen grains depending on the species (Bernhardt *et al.* 1984) matching or exceeding ovule number in the ovary (Kenrick and Knox 1982). This implies that all the seeds within a pod are full sibs. This very low pollen grain to ovule number ratio (1.2) suggests that the polyad of *Acacia* has considerable selective advantage, and is a highly efficient reproductive unit (Knox and Kenrick 1983).

Acacia tortilis is a species of arid ecosystems with poor soils in the Middle East and Africa that has considerable economic potential (Wickens *et al.* 1995). It's restoration in natural habitats as well as its growth in commercial plantations will be improved by knowledge of its natural history and by applied breeding programs. In tree-breeding programs of species like *A. tortilis*, economic and

logistic considerations often preclude the use of controlled crosses for estimating genetic parameters (*e.g.* heritability of traits). Instead families derived from open-pollinated seeds are commonly used to quantify genetic variances, heritability, and potential genetic gains for characteristics of interest (Namkoong 1966). Thus, detailed knowledge of the reproductive biology, which ultimately determines the genetic composition and relatedness of progeny arrays, is of paramount importance in formulating efficient tree-breeding and restoration programs (Mandal *et al.* 1994).

Majority of studies on the reproductive biology of acacias have been limited to diploid Australian species. Little is known about the mating systems of *Acacia* species native to Africa and Asia (Moran *et al.* 1989; Muona *et al.* 1991). In this paper we report a mating-system analysis of several populations of *Acacia tortilis* growing naturally in Southern Sinai, Egypt, using allozyme marker loci.

Material and Methods

Study species

Acacia tortilis (Forsk.) Hayne (subfamily Mimosoideae, family Leguminosae), one of about 135 African acacia species (NFTA 1991), is armed with thorns (often called "umbrella thorn") and produces highly palatable pods. *Acacia tortilis* consists of four subspecies: *tortilis*, *spirocarpa*, *heteracantha*, and *raddiana* (Brenan 1983). These four subspecies occur in arid and semi-arid lands of Africa and the Near East (Wickens*et al.* 1995). Only two subspecies; *tortilis* and *raddiana* occur in Egypt (Boulos 1999) with only subspecies *raddiana* in the study area (Halevy and Orshan 1972). As with other African acacias, *A. tortilis* is a polyploid complex (Fagg 1991), most are tetraploids ($2n=4x=52$) but ssp. *raddiana* is an octoploid ($2n=8x=104$).

Acacia tortilis varies from multi-stemmed shrubs (ssp. *tortilis*), to trees up to 20 m tall with rounded (ssp. *raddiana*) or flat-topped (ssp. *heteracantha* and *spirocarpa*) crowns (Danida Forest Seed Centre 2000). Flowering is prolific with up to 400 flowers/meter of branch. Flowers are typically protandrous, and individual inflorescences last for a single day (Stone *et al.* 1996; Tybirk 1989; 1993; Willmer and Stone 1997). Flowers open well before dawn and release their pollen at or shortly after sunrise (Stone *et al.* 1998). Flowers produce minute quantities of highly concentrated nectar (Stone *et al.* 1996) and receive substantial visitation from nectar foraging insects including megachilid and halictid bees (Stone *et al.* 1998). Flowers later develop into bunches of spirally twisted pods. Fruits are yellow brown, 5-15 cm long, with longitudinal veins and are slightly constricted between the seeds. Pods are semi-dehiscent, *i.e.* mature pods open but remain on the tree without releasing the seeds (Danida Forest Seed Centre 2000).

Acacia tortilis is the most widespread, drought-resistant, and heat-tolerant acacia among the twenty five species growing in the Middle East (Halevy and Orshan 1972; Zohary 1973). It grows in desert wadis and sandy plains, usually in water catchment areas, and is common along the Red Sea coast, Eastern Desert, Gebel Elba, and Sinai (Boulos 1999). It dominates mainly rocky wadi-bed habitats near the foothills and sometimes the slopes of metamorphic mountains in Southern Sinai (Moustafa *et al.* 1998). The main Sinai localities are Wadi Feiran and its tributaries, W. Mandar, W. Lithi, W. Solaf, W. El-Nasb, Ladid area, and El-Qaa plain. The species is threatened in the Sinai due to prolonged drought and human interference leading to increased mortality of mature trees and dramatically reduced natural recruitment (Abd El-Wahab 1995; Moustafa *et al.* 2000).

Seed collection and germination

Acacia tortilis ssp. *raddiana* has two cycles of flowering and fruiting each year. The main (and more robust) fruiting period is in June and July while the second occurs in February. Twelve *A. tortilis* ssp. *raddiana* populations were sampled in the summer from two wadi systems in South Eastern Sinai, Egypt (Fig.1) six populations from Wadi Mandar and six populations from Wadi Lithi. The minimum distance between two near populations was 1-2 km (between population M1 & M2, L2 & L3, and L5 & L6). Two populations were represented by 21 seed families, four by 10-18, and six by 3-8 (Table 1). The number of sampled families was insured to be representative and depended on the population size, number of reproductive trees, and seed availability.

Seeds were chosen at random from bulk open-pollinated seeds from each tree. Seeds were treated with 95% sulfuric acid for 25-30 min to scarify the impervious seed coat and then rinsed thoroughly with water. They were sown on Fafard mix no.3B and placed in a greenhouse (Plant Biology Department, University of Georgia) to grow to 5-10 cm in height. Up to twelve progeny from each family were crushed and used in gel electrophoresis.

Electrophoresis

Enzymes were extracted by manually crushing the seedling in a pre-cooled mortar and pestle using Wendel and Parks (1982) extraction buffer. Starch-gel electrophoresis (10% starch gels) was used to resolve isozyme electromorphs. Gels were stained for six enzyme systems to resolve 7 polymorphic loci: Menadione reductase (Mnr), Phosphoglucoisomerase (Pgi), Phosphoglucomutase (Pgm), Fluorescent esterase (Fe, two loci), Diaphorase (Dia), and Malate dehydrogenase (Mdh). Two buffer systems of Soltis *et al.* (1983) were used to resolve the enzymes; System 6 (Mnr, Pgi, Pgm, Fe), and a modified system 8 (Dia). Morpholine citrate (MC) which comes from Conkle *et al.* (1982) was used to resolve Mdh. Enzymes were visualized using standard colorimetric staining methods (Vallejos 1983). Stain recipes were from Soltis *et al.* (1983) except for Mnr and Dia, which were from Cheliak and Pitel (1984).

TABLE 1. Population mating system parameters including multilocus outcrossing rates (t_m), single locus outcrossing rate (t_s), $t_m - t_s$, correlations of outcrossed paternity (r_p), and the effective number of pollen parents (N_{ep}).

| Location	Population	No. families	No. of progeny	t_m (SD)	t_s (SD)	$t_m - t_s$ (SD)	r_{pm} (SD)	N_{ep}
Wadi Mandar	M1	13	109	0.994 (0.088)	0.943 (0.112)	0.051 (0.062)	0.104 (0.284)	9.6
	M2	8	72	1.143 (0.086)	1.193 (0.075)	-0.050 (0.070)	0.128 (0.256)	7.8
	M3	18	162	1.082 (0.071)	1.036 (0.076)	0.046 (0.050)	0.087 (0.153)	11.5
	M4	3	36	1.200 (0.268)	1.200 (0.267)	0.000 (0.057)	0.031 (0.034)	32.3
	M5	21	190	1.039 (0.070)	1.152 (0.049)	-0.113 (0.056)	0.105 (0.266)	9.5
	M6	21	203	1.050 (0.061)	1.200 (0.067)	-0.150 (0.093)	0.171 (0.244)	5.9
	Mean/Total	84	771	1.085 (0.075)	1.121 (0.107)	-0.036 (0.083)	0.104 (0.046)	12.8
Wadi Lithi	L1	10	47	1.200 (0.020)	1.200 (0.030)	0.000 (0.033)	0.657 (0.351)	1.52
	L2	5	42	1.200 (0.068)	1.163 (0.081)	0.037 (0.049)	0.100 (0.151)	10.0
	L3	8	83	1.045 (0.085)	1.107 (0.068)	-0.062 (0.068)	0.060 (0.115)	16.7
	L4	5	59	1.010 (0.140)	1.179 (0.128)	-0.169 (0.074)	0.043 (0.054)	23.3
	L5	12	110	1.200 (0.004)	1.200 (0.022)	0.000 (0.021)	0.094 (0.262)	10.6
	L6	3	28	1.109 (0.258)	1.150 (0.269)	-0.041 (0.055)	0.058 (0.062)	17.2
	Mean/Total	43	369	1.127 (0.086)	1.167 (0.035)	-0.039 (0.050)	0.169 (0.240)	13.2
Mean/Total	12	127	1141	1.106 (0.080)	1.144 (0.080)	-0.038 (0.074)	0.137 (0.168)	13.00
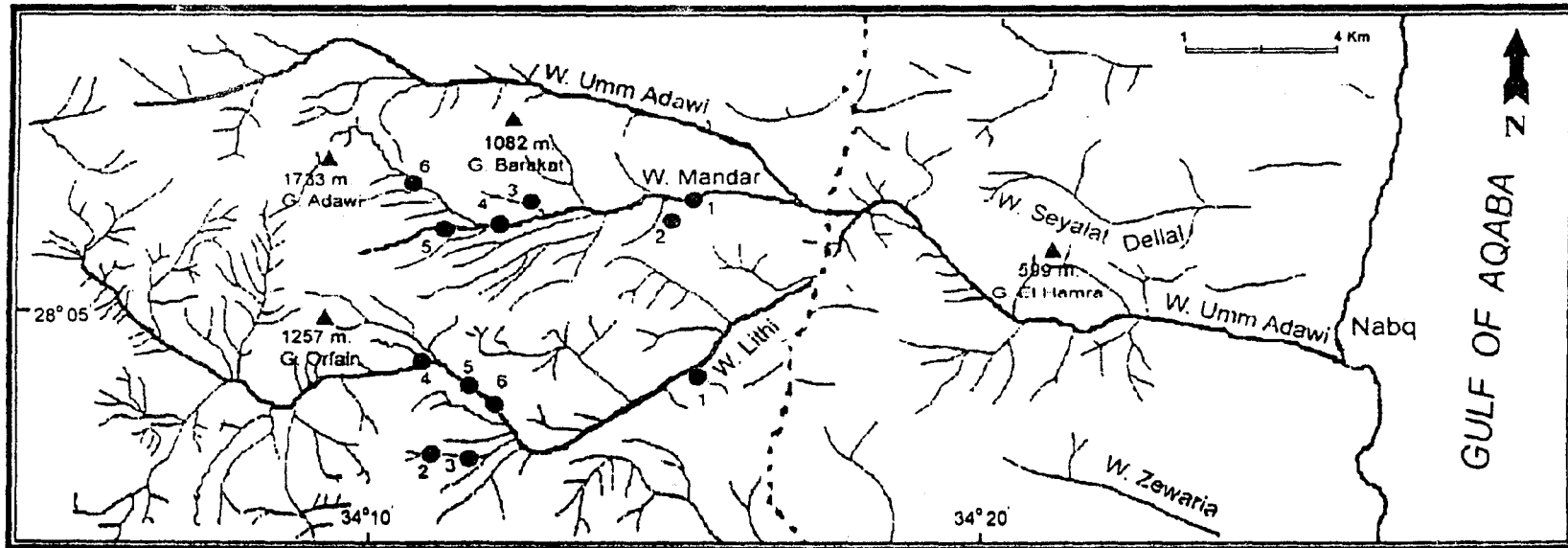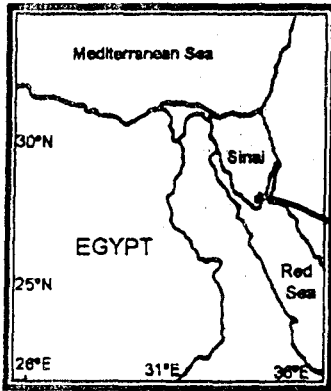
Fig. 1. Location map of the studied populations in Wadi Mandar and Wadi Lithi basins. The population numbers correspond to the numbers in Table 1.

Mating-system analysis

Multilocus (t_m) and single-locus (t_s) outcrossing rates and pollen allele frequencies were estimated for each population using maximum likelihood procedures. These parameters were estimated using the multilocus mating system program MLTR (Multilocus t and r) version 3 for Windows (Ritland 2002), based on the multilocus mixed-mating model of Ritland and Jain (1981). Standard errors for all outcrossing rates and allele frequencies were calculated from 1000 bootstrap replicates. Pollen gene frequencies were not constrained to equal ovule frequencies for the estimation of outcrossing rates.

The correlation of outcrossed paternity (r_p) was estimated according to the sibling-pair correlated mating model of Ritland (1989). The number of pollen donors contributing to a family, was estimated as the reciprocal of r_p (Ritland 1989). Inbreeding coefficients for maternal individuals (F_M) were estimated for each population using maximum likelihood procedures (Ritland 2002). Inbreeding coefficients of mothers (F_M) were compared with an equilibrium inbreeding coefficient (F_{eq}) to determine if adult populations were in inbreeding equilibrium.. The equilibrium value of the inbreeding coefficient (F_{eq}) was calculated as $F_{eq} = (1 - t_m)/(1 + t_m)$ (Fyfe and Bailey 1951). F_{eq} represents the level of inbreeding expected from selfing alone under the mixed mating model (Neel et al. 2001). Levels of biparental inbreeding were estimated as the difference between the mean single locus estimate of outcrossing (t_s) and the multilocus estimate (t_m) (Brown 1979).

Results

Allozyme polymorphisms

Even though *Acacia tortilis* subspecies *raddiana* is a polypoid, the observed inheritance pattern was consistent with diploid inheritance. Six enzyme systems were stained clearly and were encoded by seven putative loci. All enzymes migrated anodally. The total number of alleles in each population for the seven polymorphic loci ranged from 17 (L1 and L2) to 22 (M6) with a mean of 18.8.

Population mating-system parameters

Population t_m estimates ranged from 0.99 to 1.2 (Table 1). The population with the lowest outcrossing rate was M1 (0.994 ± 0.088) and the highest was L5 (1.2 ± 0.004). Three other populations (M4, L1, and L2) also had t_m = 1.2 but with higher SD values. The mean outcrossing rate (t_m) over all the *A. tortilis* populations was 1.106 ± 0.08. At Wadi Mandar, the outcrossing rate ranged from 0.994 ± 0.088 (M1) to 1.2 ± 0.268 (M4) with a mean of 1.085 ± 0.075. Values of t_m ranged from 1.01 ± 0.14 to 1.2 ± 0.004 with a mean of 1.127 ± 0.086 at Wadi Lithi. Population single locus outcrossing rates (t_s) ranged from 0.943 ± 0.112 (M1) to 1.2 ± 0.022 (L5) with an overall mean of 1.144 ± 0.080 (Table 1). Also, the mean t_s was a little higher at Wadi Lithi (1.16 ± 0.035) than that at Wadi Mandar (1.12 ± 0.107).

Multilocus correlations of outcrossed paternity (r_{pm}) were not significantly different than zero in all populations. It ranged from 0.031 ± 0.034 to 0.657 ± 0.351 with an overall population mean of 0.137 ± 0.168. While the mean at Wadi Mandar was 0.104, the mean r_{pm} was somewhat higher 0.169 ± 0.240 at Wadi Lithi. These data indicate that more than one father contributed to individual progeny arrays in all populations with a mean range from 1.5 to 32.3 fathers per progeny array. These N_{ep} estimates exceeded the sample size within some populations and even, sometimes (e.g M4, L2, and L3), it exceeded the actual population size indicating that these populations are experiencing gene immigration from other populations.

Inbreeding coefficient and biparental inbreeding estimates

Inbreeding coefficients of maternal individuals (F_M) and equilibrium values of the inbreeding coefficient (F_{eq}) were not significantly higher than zero for all *Acacia tortilis* populations. Estimates of biparental inbreeding (t_m-t_s) were also not significantly greater than zero in all populations indicating that there is almost no biparental inbreeding in *A. tortilis* populations. Although population t_m and t_s were exactly the same in two populations (M4 and L5), t_s was higher than t_m in six populations (M2, M5, M6, L3, L4, and L6).

Discussion

Three aspects of the mating system may make major contributions to nonrandom mating in populations and thus have direct application to conservation: selfing rate, amount of biparental inbreeding, and correlation of outcrossed paternity (Neel *et al.* 2001). Most forest tree species experience high levels of outcrossing (Muona 1990), and *Acacia* species are no exception (Bernhardt 1987; Stone *et al.* 1996; Tybirk 1989; 1993). However, Olng'otie (1991) estimated the mean outcrossing rate for ten African populations of *Acacia tortilis* as 0.35 suggesting that the species is self-compatible. Our estimates differ substantially and indicate that *A. tortilis* sp. *raddiana* is predominantly outcrossing, suggesting the presence of a strong self-incompatibility system. These results are comparable to those obtained by Bernhardt *et al.* (1984) who showed that self-sterility in *A. retinodes* is almost complete, and Moran *et al.* (1989) who found that two tropical acacias, *A. auriculiformes* and *A. crassicarpa*, were primarily outcrossing. Studies on other acacia species indicated that they are primarily insect-pollinated, outcrossing and show varying degrees of self-incompatibility (Bernhardt *et at.* 1984; Kenrick and Knox 1985; Moran *et al.* 1989; Muona *et al.* 1991). In contrast, Mandal *et al.* (1994) acknowledging the difficulties and shortcomings associated with the analysis of polyploids gave a best estimate of the outcrossing rate of *A. nilotica* ssp. *leiocarpa* as $t_m = 0.38$.

Arroyo (1981) noted that the basic unit of reproduction in the Mimosoideae is the inflorescence not the individual flower, because the flowers tend to be tiny, numerous, and densely massed. There are three floral mechanisms in acacias

which promote outcrossing. Many species show protogynous dichogamy with the stigma receptive to pollen before the pollen is released from the anther (Bernhardt *et al.* 1984). Andromonoecy with male and hermaphrodite flowers on the same plant also promotes pollen transfer from flower to flower (Sinha 1971) as does self-incompatibility (Kenrick and Knox 1985; Zapata and Arroyo 1978).

Inbreeding coefficients of the maternal individuals (F_M) were not significantly greater than zero in all populations and comparisons of F_M and the expected equilibrium inbreeding coefficient were in general not significantly different. Biparental inbreeding is typically less severe than inbreeding from selfing, but may still cause substantial inbreeding depression (Nason and Ellstrand 1995). Unlike outcrossing rates, only indirect estimates of the level of biparental inbreeding (as t_m-t_s) are possible. With biparental inbreeding the single-locus outcrossing rate is less than t_m (Ritland 1990; Shaw *et al.* 1981). Our results, indicate that biparental inbreeding is not an important factor on these *A. tortlis* populations since the values of t_m-t_s were not above zero in any population.

Deviations from random mating can occur if outcrossed siblings are sired by the same father (Ritland and El-Kassaby 1985). This mating pattern can be estimated by the correlation of outcrossed paternity (Ritland 1989), which is the proportion of randomly chosen outcrossed progeny pairs that are full sibs. Estimates of correlation of outcrossed paternity (r_p) indicated that multiple fathers contributed to individual progeny arrays in all populations. This means that outcrossing in *A. tortilis* is approximately random. As a result, any conservation plan for *A. tortilis* should strive to maintain random mating in populations by insuring adequate levels of pollination. Also, if restoration is undertaken, efforts should be made to include genetically unrelated individuals to prevent inbreeding. Further research using paternity analysis to describe breeding patterns within populations could provide valuable insights into pollinator behavior, and rates of gene flow among populations, and thus, would have direct conservation implications.

The mating system has a major influence on the structuring of genetic variation within a species (*e.g.* Gottlieb 1973; Moran and Brown 1980). Highly outcrossing species typically have most of their genetic diversity residing within populations with relatively low differentiation among populations while inbreeding taxa show the opposite pattern. Zaghloul *et al.* (2007) showed that a high proportion (96%) of the genetic variation in *Acacia tortilis* ssp. *raddiana* populations resides within populations. This result is consistent with the rates of outcrossing observed in this study and may be indicative of relatively high historical levels of gene flow among populations. It seems, then, that mating patterns have contributed to the maintenance of genetic diversity within populations of *A. tortilis*. This information has bearing on conservation priorities for *in situ* conservation and on collection (Brown and Briggs 1991) and breeding strategies (Ritland and El-Kasaby 1985) for *ex situ* conservation. Genetic

diversity in a highly outbreeding taxon such as *Acacia tortilis* could, therefore, be effectively conserved by maintaining a few populations of sufficient size to be protected from the effects of genetic drift. Additional populations would provide protection from catastrophic events but are not likely to capture additional genetic diversity (e.g. Brown 1989).

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صونْ عشـائر السيـال *Acacia tortilis* subsp. *raddiana* فى جنوب سيناء ، مصر. ٢ـ تحليل نظام التزاوج

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تتعرض أشجار السيال *Acacia tortilis* subsp. *raddiana*، وهو نبات طبيعى ينمو فى الموائل شديدة القحولة ذات التربة الفقيرة فى الشرق الأوسط وأفريقيا، إلى إنخفاض فى حجم وعدد وتواصل عشائره نتيجة للأنشطة الإنسانية الغير منظمة. وبمعرفة التاريخ الطبيعى لهذه العشائر وبواسطة برامج الإكثار يمكن تحسين فرص إعادة تأهيل هذا النبات فى مواطنه الطبيعية ورفع معدلات نموه فى المزارع الإقتصادية.

تقدم هذه الدراسة تحليلا لمعدلات التزاوج الخلطى والبينى فى نبات السيال النامى فى منطقة جنوب سيناء وذلك للإستخدامات المهمة المترتبة على هذا، حيث أنه يتيح تصميم وتنفيذ برامج وإستراتيجيات صون وإعادة تأهيل لعشائر هذا النبات أكثر فعالية. وفى إطار هذا الهدف ، تم خلال هذا البحث جمع بذور من مائة وسبعة وعشرون شجرة ممثلة لإثنى عشرة عشيرة منتشرة فى منطقة الدراسة. وتم رصد سبعة إنزيمات أليلية متباينة وذلك لتقدير معدلات التلقيح الخلطى ومعدلات التزاوج البينى الناتج عن نفس الآباء biparental inbreeding، بالإضافة إلى العدد المؤثر لواهبى حبوب اللقاح لكل شجرة أم.

هذا ولقد أوضحت الدراسة أن متوسط التلقيح الخلطى t_m على مستوى العشائر كلها كان ١,١٠٦ ± ٠,٠٨. وأن العدد المؤثر من واهبى حبوب اللقاح لكل شجرة أم كان عاليا، مشيرا إلى معدل منخفض للتزاوج البينى وثيق الصلة داخل كل شجرة، بينما كانت معدلات التزاوج البينى الناتج عن نفس الآباء لا تزيد عن الصفر إحصائيا فى كل العشائر.

مما سبق يتضح أن عشائر أشجار السيال ممكن صونها بشكل فعال عن طريق صون عدد قليل من العشائر ذات الحجم الكبير وذلك لتقليل الفقد فى التنوع الوراثى وللحفاظ على مجتمعات الملقحات التى بواسطتها يتم التلقيح الخلطى وتدفق الجينات بين العشائر المختلفة. وأن أية مجهودات وأنشطة إعادة تأهيل يجب أن تشمل أكبر عدد ممكن من الأفراد البعيدين وراثيا وذلك لتجنب أية زيادة فى التزاوج البينى.