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Population substructure in *Alkanna orientalis* (Boraginaceae) in the Sinai Desert, in relation to its pollinator behaviour

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Abstract

Subpopulation genetic structure was studied in a population of the short-lived perennial plant *Alkanna orientalis* from the Sinai Desert, Egypt. The population investigated was subdivided for sampling into four subpopulations, which were located within three steep-sided wadis and a central plain area. Results from previous studies suggested that bee pollinator behaviour was likely to cause limited gene dispersal within the population and that subpopulations might have diverged from each other genetically. Seven RAPD primers were used to detect polymorphisms in the population. Differences between subpopulations in fragment frequency were found for several of the 45 polymorphic RAPD fragments scored. Population subdivision was evident from cluster analysis, and an analysis of genetic distances showed that there was significant genetic differentiation between all subpopulations. Nevertheless, more extensive gene flow appears to take place within the population than was expected, as demonstrated by a higher level of genetic similarity between subpopulations from two of the narrow wadis and the interconnecting plain. It is suggested that seed transport mediated by periodic flash floods is responsible for this pattern.

Keywords: population structure, pollination, seed dispersal, RAPDs, *Alkanna orientalis*, genetic variability

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Introduction

The pollination biology and mating system of a plant species are expected to have significant effects on its population genetic structure (Schaal & Levin 1976; Levin 1979; Turner *et al*. 1982; Handel 1985; Hamrick & Godt 1989). From a review of the literature, Govindaraju (1988) showed that predominantly self-pollinating species tend to exhibit high levels of genetic variation between populations (as measured by G_{ST}), whereas wind-pollinated species exhibit low levels of variation among populations, and insect-pollinated plants show intermediate levels. These differences are presumed to reflect the extent of gene dispersal between populations, which is likely to be more closely correlated with pollen flow than seed movement.

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In insect-pollinated species, levels of pollen flow within a population will be greatly affected by the flight behaviour of the pollinator. For example, Schmitt (1980) showed that the flight distance of bumble bees between *Senecio* plants was low relative to that of butterflies and, in theory, this should result in a smaller neighbourhood size and greater population genetic substructure in populations worked by bees. Despite the accepted importance of pollinator behaviour for the genetic structure of plant populations, there have been few studies which have analysed population substructure in wild plant populations pollinated by insects of known flight behaviour. Moreover, the relative importance of pollen and seed dispersal on gene flow and population substructure has seldom been studied in detail (but see McCauley 1995; Ennos 1994).

Here we report an analysis of population substructure using RAPDs within a population of the plant *Alkanna orientalis* (L.) Boiss. (Boraginaceae) located near the town of St Catherine in the Sinai Desert, Egypt. Previous studies

by Willmer *et al*. (1994) and Gilbert *et al*. (1996) have provided a detailed background of the behaviour of the main pollinator of *A. orientalis* growing at this location, and of morphological variation between subpopulations of the plant.

Alkanna orientalis occurs at elevations of ≈ 1500 m and above in the Sinai Mountains. It is a short-lived perennial that grows to 1 m in diameter and usually flowers in its second year. Up to four seeds can be produced per fruit, although in the field a seed set of around 20% is reached (Gilbert *et al*. 1996). Its leaves and flowers are covered with sticky, irritant, glandular hairs, protecting them from extensive herbivory. The plant flowers from the end of March until the beginning of June and produces about 10–60 flowers per plant, although up to 700 flowers have been recorded on some older plants. Flowers are 1–2 cm long, golden-yellow and trumpet-shaped (Gilbert *et al*. 1996). Reproduction of this self-incompatible species is entirely sexual (Gilbert *et al*. 1996). Flowers are open for 1 or 2 days, after which they turn pale yellow or white and/or wither. Subpopulations are fairly large, from around 50 plants in Wadi Tofaha to several hundred in the other subpopulations.

During April, *A. orientalis* is one of very few plants flowering at the location studied, and its main visitor is the solitary bee *Anthophora pauperata* (Apoidea, Anthophoridae) (Willmer *et al*. 1994; Gilbert *et al*. 1996). As other plant species begin to flower, the bee transfers its attention away from *A. orientalis*, which then becomes visited by hoverflies and bombyliids (Gilbert *et al*. 1996). These insects are probably less effective as pollinators, and *A. pauperata* is regarded as the main pollinator of *A. orientalis* (Gilbert *et al*. 1996).

Willmer *et al*. (1994) reported that males of *A. pauperata* patrol a territory, a clump of *A. orientalis* plants, not only when the females are out of their nests, which peaks in early morning and afternoon, but also in the interim period. This territorial behaviour was reported to be strongest in the narrow wadis at St Catherine, where floral rewards and bee density are high, but was rarely observed in the more open plain habitat at the outflow of the wadis. The sizes of bee territories ranged from 5×5 m to 15×15 m, with each containing from three to nine clumps of plants and an average of 300 open flowers on a given day. Gilbert *et al*. (1996) studied several morphological and ecological characters of plants from the same sites in the Sinai population as were used in the present study. Characters considered to be important for the pollinator behaviour were analysed, namely flower size and structure, nectar availability, seed production, flower phenology, and numbers of flowers open. Differences between the subpopulations were observed, implying either population substructuring or environmental modification of characters.

It was postulated from these observations that gene flow by pollinator movement would be highly restricted within the St Catherine population of *A. orientalis* (Willmer *et al*. 1994) and that this might bring about genetic divergence between subpopulations. The present study was conducted to test this hypothesis.

Materials and methods

Description of the sampling sites

Plant material was collected in 1994 from a population of *Alkanna orientalis* that grew in the area around the field station of Suez Canal University at St Catherine (1641 m a.s.l.) in the southern Sinai Desert of Egypt. The area is surrounded by mountains rising up to 2400 m and comprises a broad open area, hereafter referred to as the Plain (Fig. 1) and several narrow steep-sided wadis which radiate from the Plain. The sampled river valleys, dry for most of the year, are named Wadi Arbaein, Wadi Dir and Wadi Tofaha. The wadis are largely isolated from each other by their high sides, although they are interconnected at their outflow via the open Plain. Wadi Tofaha is the shortest, narrowest and steepest of the three wadis, whereas Wadi Dir is broader at the point where it meets the Plain and is (subjectively) less isolated from the Plain area with its broader entrance and less steep sides. Wadi Arbaein is intermediate in regard to both its breadth and the steepness of its sides. Plants in the wadis occur in high numbers (100 or more) up to 30 m above the wadi bed and can also be found at much higher elevations in the surrounding mountains. Densities of the subpopulations in the wadis are from 235 to 653 plants per ha (Gilbert *et al*. 1996). Leaf material was collected from randomly chosen plants on the Plain and up to 2 km into the wadis from the Plain, mainly from the base of the wadis. In each case the leaves were collected from only one branch of a plant. The number of plants analysed was seven for Wadi Tofaha, nine for Wadi Arbaein, 16 for Wadi Dir and seven for the Plain subpopulation.

Plant collection, DNA extraction and RAPD analysis

Plant leaves were collected and dried with silica gel and thereafter kept at –20 °C. DNA was extracted using a standard CTAB extraction procedure (Wolff *et al*. 1994; modified after Saghai-Maroof *et al*. 1984). An additional ammonium acetate purification step was used (Weising *et al*. 1995).

RAPD analysis was performed in 25-μL volume reactions according to Wolff & Peters-Van Rijn (1993). Amplifications were carried out in an MJ Research PTC 100 thermocycler, which was programmed according to Wolff (1996). A 100-fold range of DNA concentrations gave different RAPD patterns, possibly caused by impurities in the DNA. Therefore, an additional gel purification

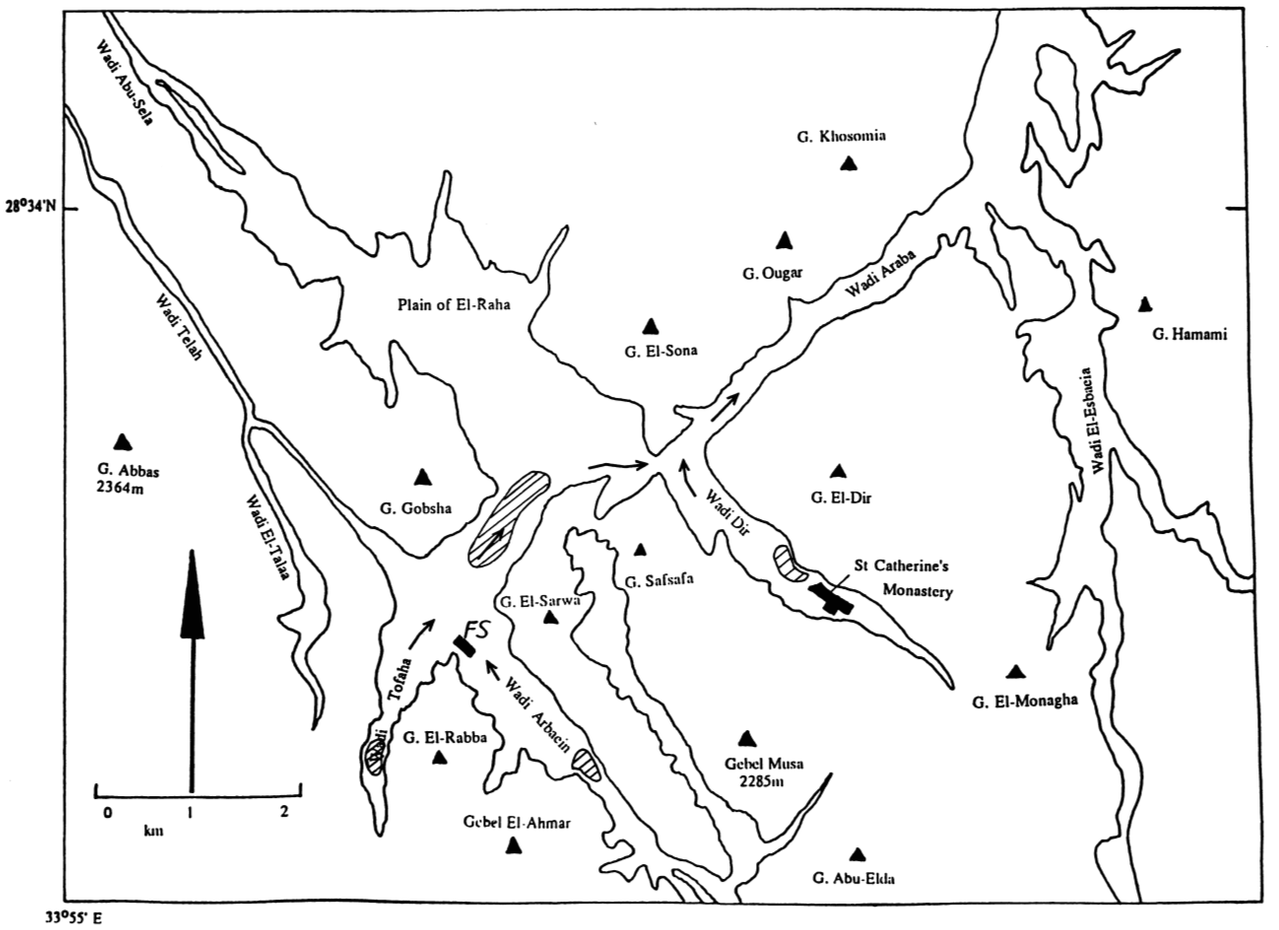

Fig. 1 Location of the study population of *Alkanna orientalis* in the Sinai Desert (from Gilbert *et al*. 1996). The arrows represent the direction of flash floods and hatched areas are the areas from which the plants were collected

was performed. The DNA was run on a 1% low melting agarose gel, made with 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). After a run of ≈ 1.5 h (8 V/cm), the high-molecular-weight DNA was excised from the gel. Eppendorf tubes with the gel fragments (each ≈ 250 mg) were incubated in a waterbath of 70 °C to melt the agarose. Two-hundred and fifty microlitres TE (10 mM Tris, 1 mM EDTA, pH 8.0) were added and the solution was mixed after incubation at 70 °C. Five-hundred microlitres of phenol were added and the solution was vortexed. After spinning for 4 min at 16 000 *g*, the upper layer was pipetted into fresh tubes. Five-hundred microlitres of chloroform/isoamylalcohol (24 : 1 ratio) were added and the solution was vortexed, spun and separated as before. To each tube 50 µL of sodium acetate (2.5 M, pH 5) and 350 µL cold isopropanol were added. Tubes were inverted several times and stored at –20 °C for 15 min. They were then spun in a microfuge for 10 min (16 000 *g*) and the supernatant was decanted. The DNA pellet was washed with 70% ethanol by flicking the tube. After spinning the tube for 10 min and decanting the supernatant, the pellet was dried at room temperature for 15 min and dissolved in TE.

The gel-purified DNA was brought to a concentration of 5 ng/µL. This DNA used in RAPD analysis gave identical RAPD patterns in a 100-fold range of DNA concentrations. The gels were photographed for analysis. Nineteen primers from the OPH set were tested. The seven primers that gave the clearest banding patterns were used and phenotypic frequencies of 45 polymorphic bands were scored (Table 1). Plants that had a missing value for any of the primers were not included in the analysis. A table with absence/presence of the fragments is available upon request from the corresponding author.

Statistics

The resulting presence/absence fragment matrix was analysed using several computer programs. RAPDistance Version 1.03 (Armstrong *et al*. 1994) was used to construct a neighbour joining (NJ) tree of all individuals using Euclidean distances computed between all pairs of individuals.

Table 1 Phenotypic frequencies of RAPD fragments in four subpopulations of *Alkanna orientalis*, with the sample sizes in parentheses

	OPH1: GGT CGG AGA A													OPH3: AGA CGT CCA C			
Pop.	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4
Arbaein (9)	0	0.89	0	0.67	1.0	0.11	0.11	0.67	0.22	0.67	0.11	0.78	0.11	0.78	0.11	0	0.56
Dir (16)	0.06	0.31	0.06	0.25	0.88	0	0.06	0	0.06	0.12	0.06	0.56	0.06	0.88	0	0	0.38
Plain (7)	0	0.57	0.29	0.14	0.86	0	0.14	0	0.43	0.29	0	0.43	0.43	0.71	0.29	0.29	0.71
Tofaha (7)	0	0.43	0	0.71	1.0	0	0	0.14	0.43	0.29	0.29	1.0	0.43	0.29	0.71	0	0
	OPH13: GAC GCC ACA C								OPH14: ACC AGG TTG G								
	1	2	3	4	5	6			1	2	3	4	5	6	7	8	
Arbaein (9)	0	0	0.44	1.0	0	0.44			0	0.56	0.44	0	0	0	0.78	1.0	
Dir (16)	0.62	0.62	0.25	0.81	0	0.25			0.75	1.0	0	0.06	0.75	0	0.06	0	
Plain (7)	0	0	0.14	0.29	0	0.71			0.14	1.0	0	0.43	0	0	0.43	1.0	
Tofaha (7)	0	0.14	0	0.29	0.57	0			0	0.57	0	0	0	0.43	0	1.0	
	OPH15: AAT GGC GCA G								OPH17: CAC TCT CCT C						OPH18: GAA TCG GCC A		
	1	2	3	4	5	6			1	2	3	4			1	2	3	4
Arbaein (9)	0	0	0.11	0.89	0.89	0.67			0	0.89	0	1.0			0.67	0.44	0	0
Dir (16)	0.75	0	0	0.94	0.88	0.06			0.69	0.94	0.81	0.50			0.19	0.94	0.06	0.06
Plain (7)	0.14	0	0	1.0	0.86	1.0			0.14	0.43	0.14	1.0			0.14	0.71	0	0.14
Tofaha (7)	0	0.43	0	0.57	0.57	0.57			0.43	0.57	0.43	1.0			0	0.71	0	0

Shannon's index of diversity was calculated from the frequencies of the RAPD bands within each subpopulation and also over all subpopulations (King & Schaal 1989) to obtain estimates of within subpopulation genetic diversity (H_S) and total genetic diversity in the population (H_T). The equation used was: $H = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of a band.

Genetic distances were calculated between subpopulations (Phi_{ST} values) and significant differences in genetic variation between subpopulations were tested using the program AMOVA (Excoffier *et al*. 1992). This program is designed for haploid data, but it has been successfully used for RAPD data (Huff *et al*. 1993; Yeh *et al*. 1995; Peakall *et al*. 1995; Stewart & Excoffier 1996). Euclidean distances between all pairs of individuals, within and between populations, were calculated using the program RAPDistance and were used as the input for the program AMOVA. AMOVA performed an analysis of variance on these distances, partitioning the variation into within and between subpopulation components. The significance of differences between subpopulations was tested by a non-parametric permutation procedure, using 1000 permutations. Stewart & Excoffier (1996) have shown that this method of analysis can detect subpopulation structure within small data sets of the type available from the present study.

Results

A considerable amount of genetic variation within and between subpopulations was detected by the seven RAPD primers used in the study. Some of the primers detected RAPD polymorphisms within, as well as between, subpopulations, whereas other primers showed clear differentiation between subpopulations. For example, all plants of the Wadi Dir subpopulation were found to lack fragment 8 produced by primer 14, whereas all plants from the other three subpopulations possessed this fragment (Fig. 2; Table 1).

To depict the relationships among individuals based on RAPD profile similarities, a neighbour-joining (NJ) tree was constructed from the Euclidean distances between individuals (Fig. 3). It is evident from the tree that, in general, individuals from a given subpopulation tend to cluster together and are therefore more genetically similar than individuals from different subpopulations. Thus, the Wadi Dir subpopulation is clearly genetically distinct from the other subpopulations. Although individuals from the Wadi Arbaein and Wadi Tofaha subpopulations do not cluster together, certain individuals from the Plain subpopulation group with Wadi Arbaein individuals and others group with Wadi Tofaha individuals. This suggests that there is considerable genetic similarity between individuals in the Plain subpopulation and

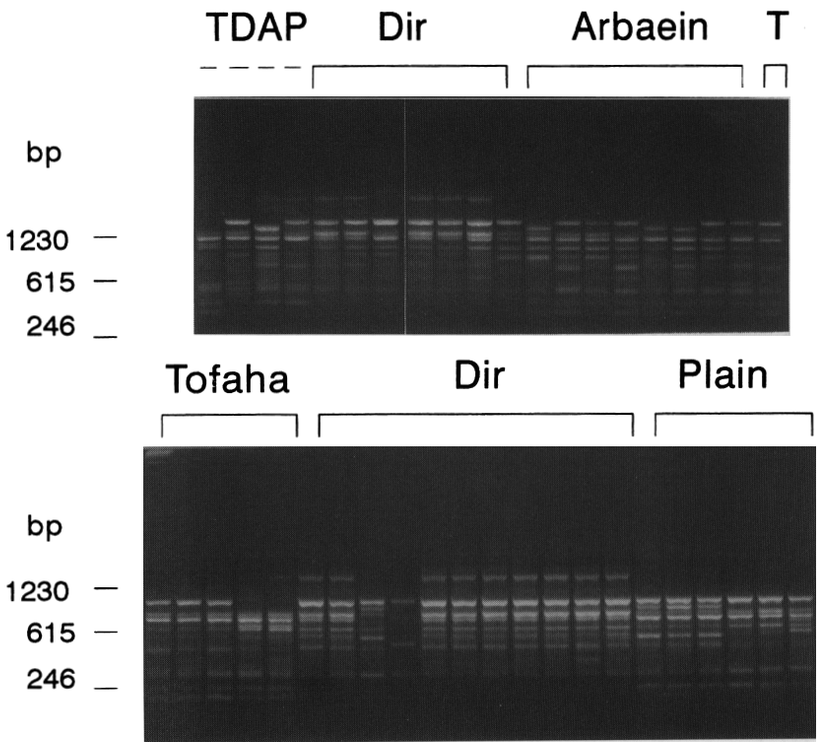

Fig. 2 Example of a RAPD pattern, obtained with primer 14. The populations are indicated with their full name or the first letter of it

certain individuals within the Wadi Arbaein and Wadi Tofaha subpopulations.

Shannon's diversity index was calculated for each subpopulation in turn (H_S for Plain = 11.54; Arbaein = 8.98; Dir = 10.41; Tofaha = 10.36), showing that subpopulations contain similar levels of diversity with an overall average of $H_S = 10.32$. Shannon's diversity index for the total population was estimated to be $H_T = 16.78$, and the proportion of total diversity distributed within populations ($H_S/H_T = 0.62$) was found to be greater than that present between subpopulations [$(H_T - H_S)/H_T = 0.38$].

The AMOVA analysis partitioned 32% of the total variation between subpopulations and 68% of the total within subpopulations, in broad agreement with the results of the Shannon's diversity index analysis. The Phi_{ST} value for all subpopulations was estimated to be 0.325, which is significantly different from zero at $P < 0.001$. All pairwise Phi_{ST} values were also significantly different from zero (Table 2). Thus, all subpopulations may be considered as significantly genetically different from each other, with the Wadi Dir subpopulation most different from the other three subpopulations, and the Plain and Wadi Tofaha subpopulations being most similar.

Discussion

The present study is one of the first studies of the population genetic substructure of an insect-pollinated desert plant. The RAPD analysis of dried leaf material from *A. orientalis* revealed that subpopulations of the species located in different wadis or on the central plain at St Catherine, South Sinai, are genetically different from each other. As it is generally believed that RAPD markers are selectively neutral, it is concluded that limited gene flow and drift are the main factors that have led to the evolution of these genetic differences. In the majority of the studies surveying the inheritance of RAPD fragments it has been shown that they are inherited in a Mendelian fashion. Furthermore, Rieseberg (1996) showed in an interspecific study that some 90% of bands of similar size are homologous. Therefore, we have assumed that RAPD fragments in *A. orientalis* are heritable and homologous if of similar size.

Nevo *et al*. (1994) and Dawson *et al*. (1993) have investigated the population genetic structure of the predominantly selfing species *Aegilops peregrina* and *Hordeum spontaneum*, respectively, from Israel. They found that significant genetic differentiation existed between populations, with specific allozymes and RAPD fragments associated with soil types or aridity stress. Because both species are highly inbreeding, these associations between molecular markers (which are assumed to be neutral to the effects of selection) and ecological factors are possibly caused by high levels of linkage disequilibrium between alleles at marker loci and alleles at loci affected by selection (Hastings 1989). As *A. orientalis* is highly outcrossing, linkage disequilibrium of this type would not be expected.

The high mountain ridges between the wadis at St Catherine make movement by bees across the ridges

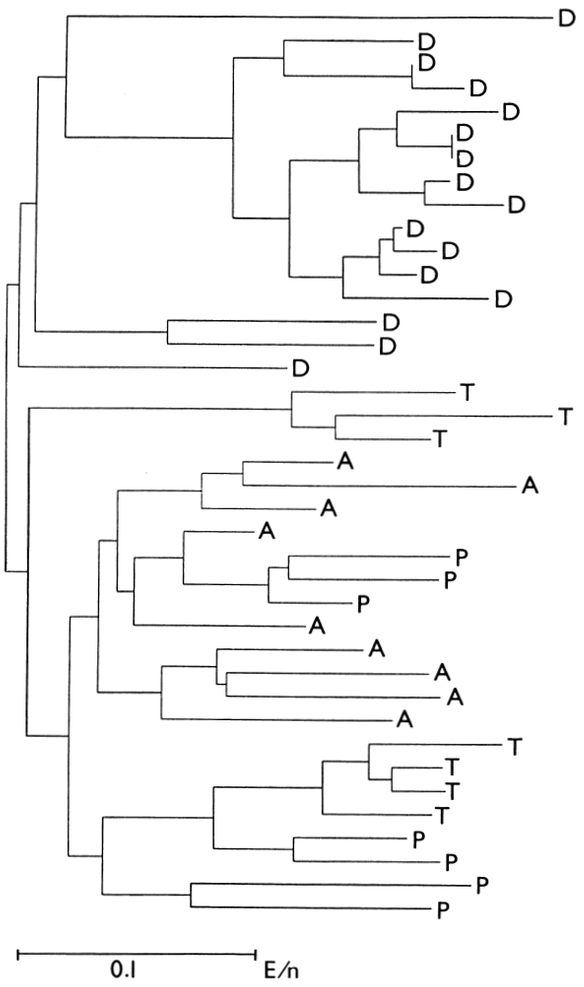

Fig.3 Neighbour-joining dendrogram of the individual *Alkanna orientalis* plants. Individuals from Wadi Dir are indicated with D, from Wadi Tofaha with T, from Wadi Arbaein with A and from Wadi Plain with P. The scale of the distances in the dendrogram is shown as E/n, where E is the Euclidean distance and n the number of polymorphic fragments

unlikely. Transport of pollen by the main bee pollinator, *Anthophora pauperata*, between wadi subpopulations via the interconnecting plain also seems unlikely, as previous studies have shown that this bee forages within a territory that is generally limited to a few plants within an area of 25–125 m^2 (Willmer *et al*. 1994; Gilbert *et al*. 1996). After *Anthophora pauperata* stops foraging on *A. orientalis*, hoverflies and bombyliids visit the flowers, but the pollinating success of these is probably limited (Gilbert *et al*. 1996).

Seed transport by flash floods, which occur very regularly in Sinai at the end of winter, seems to be a more likely cause of extensive gene flow in the population of *A. orientalis* at St Catherine. Nutlets of the species are 3–4 mm in diameter (Davis 1978) and could easily be transported by water. There are no records of ants transporting seeds of *A. orientalis*, and the distances between subpopulations (1–3 km) are too far to be covered by ants. Even in plant species with elaiosomes, ants transport seeds < 1 m (Ohkawara & Higashi 1994; Weiblen & Thomson 1995). Furthermore, if seed transport by ants was important, we might expect that gene dispersal would not be polarized in a particular direction between interconnected wadi subpopulations. The results of the RAPD analysis suggest that most gene dispersal is between the Plain subpopulation and the Wadi Tofaha and Wadi Arbaein subpopulations. The outflows of the Wadis Arbaein and Tofaha are into the Plain, and it can be envisaged therefore that gene flow via seed transport is frequent from these two wadi subpopulations into the Plain subpopulation. As Wadi Tofaha is the narrowest and steepest of the three wadis, it is very likely that, during a flash flood, transport of seeds by water would be greatest from the subpopulation in this wadi. This would account for the finding that the Plain and Wadi Tofaha subpopulations are the least genetically differentiated. The finding that the Wadi Dir subpopulation is the most genetically different of the four subpopulations also fits in with the topology of the area, reflecting the fact that plants sampled from Wadi Dir were not only most distant from all other plants sampled, but also downstream from them, with plants sampled from the Plain located nearer to Wadi Tofaha and Wadi Arbaein than to Wadi Dir. Thus, Wadi Dir is most isolated from the other subpopulations because of the direction of the stream.

Although the four subpopulations were found to contain similar high levels of RAPD diversity (as measured by Shannon's index of diversity), the Wadi Plain subpopulation contained the greatest diversity. This is also as expected if the Plain subpopulation is subject to occasional gene flow via seed transport from the Wadi Arbaein and Tofaha subpopulations.

The fact that the Plain and Wadi Tofaha subpopulations were least genetically different for RAPDs conflicts somewhat with the findings of previous comparisons between the four subpopulations for a range of floral and reproductive traits. Gilbert *et al*. (1996) reported that floral trait differences, as measured on material collected directly from the field, were greatest between the Plain and Wadi Tofaha populations and least between the Plain and

Table 2 Pairwise Phi_{ST} values between subpopulations and their significance

	Arbaein	Dir	Tofaha
Dir	0.40***	–	
Tofaha	0.29***	0.38***	–
Plain	0.20**	0.34***	0.14***

*** $P < 0.001$, ** $P < 0.01$

Wadi Dir subpopulations. This contradiction could be explained if the flower morphology differences recorded between subpopulations are, in large part, induced by the environment. Differential selection of the expression of different floral traits in the different wadis (possibly due to pollinator-mediated selection) would be expected to occur in the direction of the observed means. It is possible, however, that gene flow by flash floods might override the selection pressure of the pollinator in the Plain subpopulation and, consequently, prevent genetic differentiation for floral traits. More detailed analysis of the floral trait differences between subpopulations is required to determine whether they have a genetic basis, as this is currently unknown.

The present study indicates that the pattern of both pollen and seed flow are influential in moulding the genetic subdivision of the population of *A. orientalis* in the St Catherine area. More detailed investigation is now required of seed transport in this population to test some of the ideas put forward in this discussion. The population studied is very isolated from other populations of *A. orientalis* in the Mediterranean region, and it would be of interest to extend the genetic analysis of this species to other places where it is known to occur: Greece, Turkey and Algeria (Polunin & Huxley 1965; Polunin 1980). Such an analysis of the relationships between all of these populations and other *Alkanna* species should provide information on the biogeography of the species, and whether we are dealing with a relict population at St Catherine, which is markedly different from other populations of the species. The population from the Sinai Mountains relies mainly on *Anthophora pauperata* as its pollinator, but this population may not be representative for the species in this respect.

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The research described is part of an ongoing interest of the Universities of Cairo, St Andrews and Nottingham into the ecology of plant and insect populations in the Sinai desert. Kirsten Wolff works in various areas of molecular ecology with a special interest in the influence of mating system and genome architecture on population differentiation and speciation. Somia El-Akkad has a wide interest ranging from the physiological impact of gall formation, insecticides and pesticides on plants to population structure of desert plants. Richard Abbott studies the evolutionary biology, hybridization and biogeography of plants.
