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Genetic variability in the endemic bee *Anthophora pauperata* among wadis in the St Katherine Protectorate

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Abstract

The genetic diversity and spatial genetic population structure of the solitary bee *Anthophora pauperata* Walker 1871, a species endemic to St Katherine Protectorate, were studied by RAPD markers in seven wadis in the St Katherine Protectorate, South Sinai, Egypt. High levels of genetic diversity were found, mostly within rather than among wadis, but there were highly significant genetic differences among sites, unrelated to geographic distances between them. Reasons for these patterns may lie in the territoriality and mating behaviour of these bees.

Keywords: genetic differentiation, gene flow, RAPD

Introduction

Although evidence for the role of genetic factors in extinction is denied, or at least underplayed, by some authors (Caro & Laurenson 1994, Caughley 1994), there are authors at the other extreme who believe that many extinction events ascribed to demographic or stochastic causes actually have a largely genetic basis (Frankham 1995). Genetic causes of population decline come from a variety of influences, including the accumulation and expression of deleterious or lethal alleles or loss of fitness through lack of heterozygosity (Allendorf & Leary 1986, Mitton 1993). Such influences may arise quickly, as in the case of inbreeding depression, or they may have longer term effects, such as impeding adaptation to environmental change (Lande & Shannon 1996). Of particular concern in a rapidly changing environment is the reduced potential for evolutionary response as a result of diminished genetic variation in fragmented populations (McCauley 1993).

Genetic diversity became an issue when Frankel (1970) postulated that genetic variation is essential for the long-term survival of endangered species. For an entomophilous plant, declining abundance of its pollinator can accelerate its end, because this is frequently accompanied by a decline in genetic variability via genetic drift, which increases the likelihood of extinction (Barrett & Kohn 1991). Fragmentation and habitat destruction can add to the rate of genetic erosion by reducing gene flow between populations. The measurement or estimation of levels of genetic variation within species thus becomes critical to their conservation and management in many cases.

Random Amplified Polymorphic DNA (RAPD) markers have been used in many studies (Vandewoestijne & Baguette 2004) for assessing the habitat fragmentation effect on the genetic structure of population. Because of its ease of implementation and the small amount of material required, the RAPD technique has been recommended for this kind of work (Welsh & McCleland 1990, Williams *et al.* 1990). This study was initiated to evaluate genetic diversity using RAPDs within and between wadi subpopulations of the bee *Anthophora pauperata* Walker 1871 within the St Katherine Protectorate, the only place where it has been found. In spring *Anthophora pauperata* is the main visitor and pollinator of *Alkanna orientalis* (Willmer *et al.* 1994; Gilbert *et al.* 1996; Gilbert 1999; Stone *et al.* 1999).

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Materials & Methods

Anthophora pauperata adults fly from late March to early May, reaching their maximum around mid-April (Semida 1994). They are the earliest bees to appear in the wadis, coinciding with the early main part of the flowering of *Alkanna orientalis*; bees use *Alkanna* as a source of pollen and nectar (Zalat 1984), and flower patches are incorporated into territories by the males (Willmer *et al.* 1994). The foraging activity of female *Anthophora* is bimodal, early in the morning and in the late afternoon (Willmer *et al.* 1994; Gilbert 1999), apparently driven by daily patterns of pollen release from *Alkanna* (Stone *et al.* 1999). They appear to construct one nest cell each day (Willmer *et al.* 1994; Batra 1994).

In April 2004, individual insects were collected from seven wadis around the town of St Katherine (Fig. 1) using a conventional insect net. Captured insects were preserved at –20 ºC until subsequent treatment. The seven sampling sites were: Wadi El Arbaein, a steep rocky gorge running SW from the toan of St Katherine for 3 km (10 bees sampled); the Plain of El Raha, a flat expanse of sandy soil and rock (4); Wadi El Dir (containing the Monastery of St Catherine), broad at the point where it meets the Plain of El Raha (10); Wadi El Tofaha, the shortest, narrowest, steepest wadi, running for 1 km south from the town (10); Abu Sela, a 1.3-km long wadi at the north-western end of the Plain of El Raha (4); El Rasis, contiguous with the town of St Katherine, a broad low area 1.6 km long (3); and Wadi Telah, the longest wadi at 5.6 km, consisting of a coarse sand substrate with granite rocks and basalt dykes (10). Distances between these sites were measured as the crow flies, and also along wadi beds, using Google Earth.

DNA was extracted from the thoracic muscles using a standard CTAB extraction procedure (Wolff *et al.* 1994, modified after Saghai-Maroof *et al.* 1984); cleaning with ammonium acetate was necessary. Samples were diluted with half the volume of 7.5 M, cold ammonium acetate, cooled in a fridge for 15 min, followed by spinning for 15 min at 5000 rpm. The supernatant was taken and two volumes of cold 96% ethanol gently mixed and left for 30 min in a freezer. After spinning for 15 min, the precipitate was taken, and 500 µl of cold 70 % ethanol added for washing. The supernatant was removed and the precipitate left to air-dry at room temperature for 10-20 min, and then dissolved in a suitable volume of TE buffer. Before further analysis, it is important to determine the concentration and condition of the isolated DNA. This was done by comparison with DNA standards on concentration gels. Twenty primers from the OPH set were screened for polymorphic bands between individual insects: five primers with clear and reproducible bands were chosen for this study. The genomic DNA extracts were used as templates for PCR experiments. A reaction mixture (master mix) was prepared for each primer sufficient for all samples plus one negative control to which water was added instead of DNA. RAPD analysis was performed in 25 µl volume reactions according to Wolff & Peters Van Rijn (1993). All reagents were centrifuged and kept on ice during the preparation of the master mix. Amplifications were carried out in a Mastercycler gradient programmed according to Wolff (1996). After amplifications, samples underwent electophoresis on a 1.4% agarose gel; visualization of the DNA fragments was done in a UV cabinet unit and photographed with a Polaroid camera connected to a computer system with analytical software (GelDocu Advanced version).

RAPD bands were scored as 1 (present) or 0 (absent) using GelDocuAdvanced software. The resulting presence/absence matrix was analyzed using several computer programs. MVSP was used to construct a Neighbour Joining tree of all individuals using Euclidean distances computed between all pairs of individuals. Shannon's index of diversity ($H = -\sum p_i \ln p_i$, where p_i is the frequency of a given RAPD fragment, and ln is the natural logarithm) was calculated from the frequencies of RAPD bands within each sample and between all samples (King & Schaal 1989) to obtain estimates of the within-sample genetic

diversity (H_s) and total genetic diversity among samples (H_t). Then the proportion of diversity within samples was estimated as (H_s / H_t) and the proportion of diversity among samples as (H_t - H_s)/ H_t.

Genetic dissimilarities were calculated among samples using the routine SimPer of the Community Analysis Package 4.1.3 (Pisces Conservation Ltd, Lymington, UK) and significant differences among samples tested using Analysis of Similarity implemented by the same package. Nei's genetic distance was calculated from band frequencies as if they were alleles using the routine gnkdst from the program DISPAN by T.Ota, available as freeware. The freeware program zt (Bonnet & van der Peer 2002) was used to perform the Mantel test for a relationship between genetic and geographical distances among samples; 10000 randomisations were used.

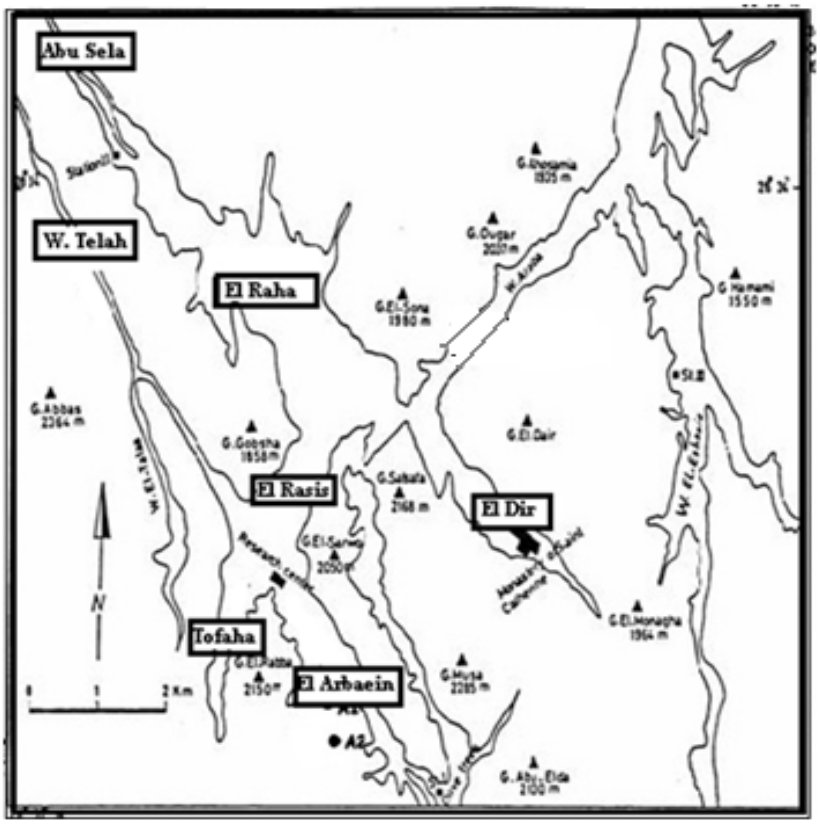

Fig. 1: Map of the area around the town of St Katherine, with collection sites marked

Results

Five oligonucleotide primers (OPH-07, OPH-12, OPH-13, OPH-15, OPH-20) were used for RAPD-PCR analysis of genomic DNA of *A. pauperata*. One hundred and three DNA fragments were generated for the whole population of samples collected from the seven sites within St Katherine Protectorate. The total number of bands scored per primer ranged from 17 (OPH-13) to 23 (OPH-15). The size of the amplified fragments ranged from 250 to 2666 base pairs (bp). Some DNA fragments (18 bands) were common in all samples, while other bands were specific for particular samples (Fig. 2). For example, wadi Telah individuals were characterized by the fragment with base pair of 520 obtained by primer 7, whereas all individuals from other samples were found to lack this fragment. Of the 103 bands analyzed, eight DNA fragments were exclusive to wadi Telah; six DNA fragments were exclusive to wadi El Dir; two DNA fragments were exclusive to El Arbaein; three fragments were

characteristic for both wadi Abu Sela and El Raha; and only one band was specific for both wadi El Tofaha and El Rasis.

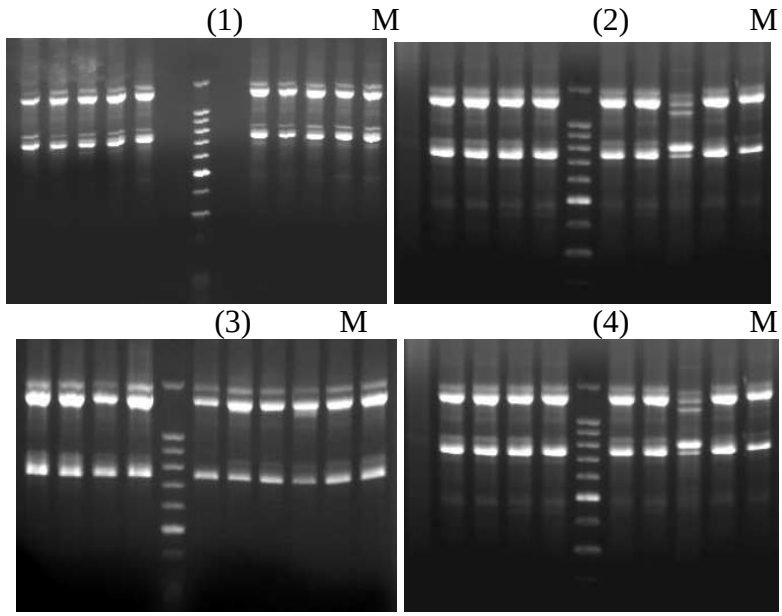

Fig. 2: RAPD pattern of *A. pauperata* using primer 7, from the following wadis around St Katherine 1: El Arbaein, 2: El Dir, 3: El Tofaha and 4: Telah. M refers to the marker with base pair from top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100

A neighbour-joining tree (Fig. 3) was constructed from the matrix of Euclidean distances between the 51 individuals collected from the seven wadis (10 individuals from each of wadi Telah, El Dir, El Arbaein, and El Tofaha, 3 from El Rasis, 4 from Abu Sela and El Raha). Most individuals from a given sample tend to cluster together, and are therefore more genetically similar than individuals from different samples, particularly individuals of El Tofaha, El Arbaein, and Abu Sela (100%), Telah (90%) and wadi El Dir (80%). Some individuals from El Rasis, El Dir and El Raha formed smaller groups.

From the frequencies of the RAPD fragments, estimates of individual genetic diversity, within-sample genetic diversity (H_s) and total genetic diversity across samples (H_t) were obtained (Table 1). The mean band diversity within individuals was between 0.84 and 0.95 of the pooled site diversity. The proportion of total diversity distributed within samples (0.917) was greater than that among samples (0.083). Analysis of similarity (Anosim) showed that sites were significantly distinct genetically (overall test statistic = 0.754, $p<0.001$), and pairwise tests showed that each was different from all others except the pair with the lowest sample sizes (Rasis and Raha).

There are highly significant differences among the sites sampled in band diversity per individual bee ($F_{6,44}$ = 14.2, $p<0.001$: Fig 4) and in the number of recorded bands per individual ($F_{6,44}$ = 13.6, $p<0.001$). Wadi El Rasis & El Dir show high mean individual band diversity, whilst Abu Sela has the lowest value.

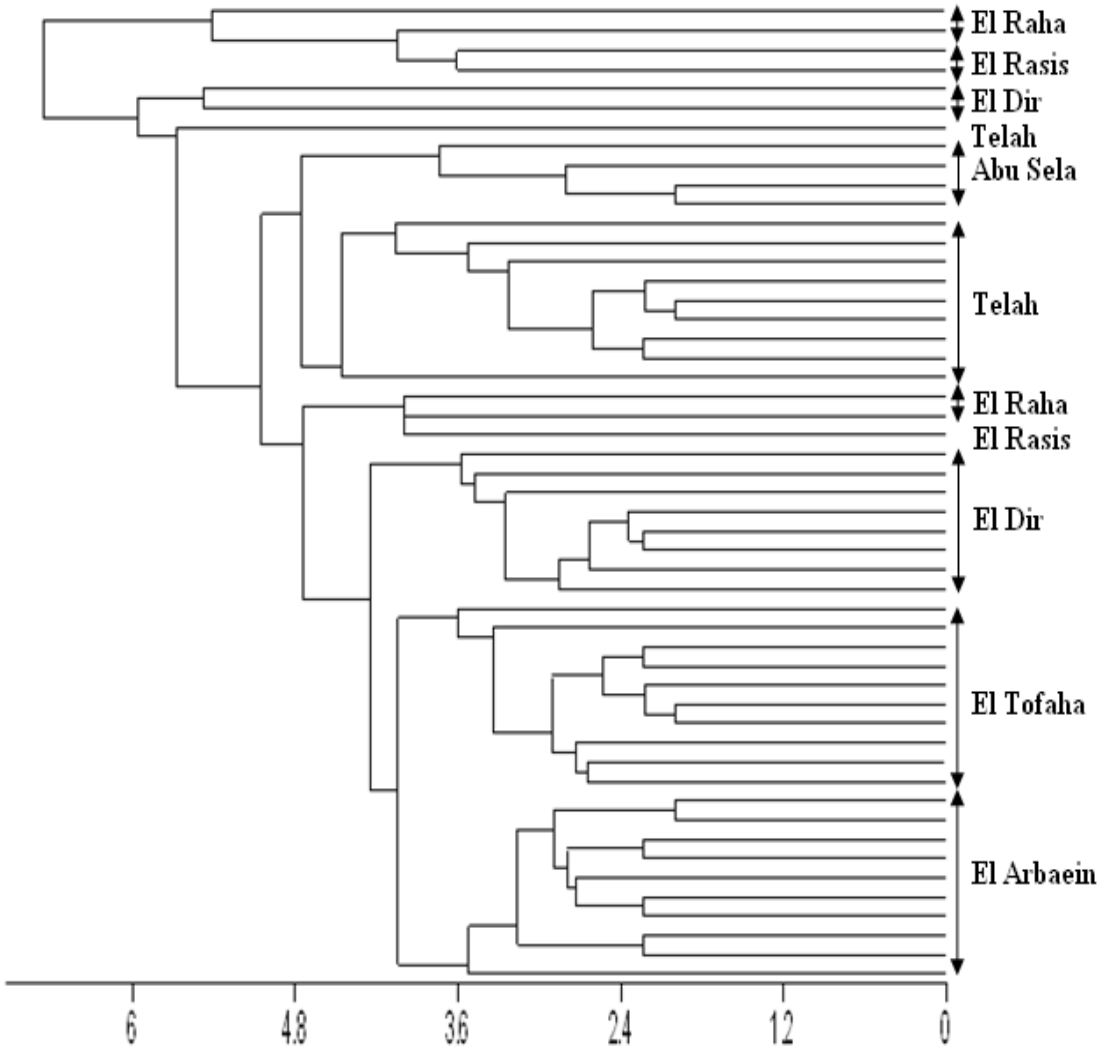

Fig. 3: Neighbour joining dendogram based on RAPD pattern of *A. pauperata*, from wadi Abu Sela, El Dir, El Tofaha El Arbaein, El Raha, El Rasis, and Telah. The scale of the distances in the dendogram is shown as E/n, where E is the Euclidean distance and n is the number of polymorphic fragments.

Estimates of genetic dissimilarities based upon 103 bands showed that the lowest average value is between individuals from wadi El Tofaha and those from wadi El Arbaein; the greatest average value is between individuals from wadi El Rasis and wadi El Abu Sela. There was no relationship between genetic dissimilarity and geographic distances either as the crow flies ($r=0.15$, $p=0.25$ one-tailed) or along wadi beds ($r=0.14$, $p=0.26$ one-tailed). A scatterplot of the pairwise genetic dissimilarity and geographical distance between sampled sites is shown in Fig. 5. Nei's genetic distances was lowest between the two poorly sampled sites (Rasis and Raha), and highest between Arbaein and Rasis. Despite the apparent differences in the extremes, genetic dissimilarity and Nei's distance were highly related (Mantel test, $r = 0.79$, $p<0.0001$). Like dissimilarity, there were no associations with geographic distance either as the crow flies ($r=0.21$, $p=0.21$) or along wadi beds ($r=0.04$, $p=0.41$).

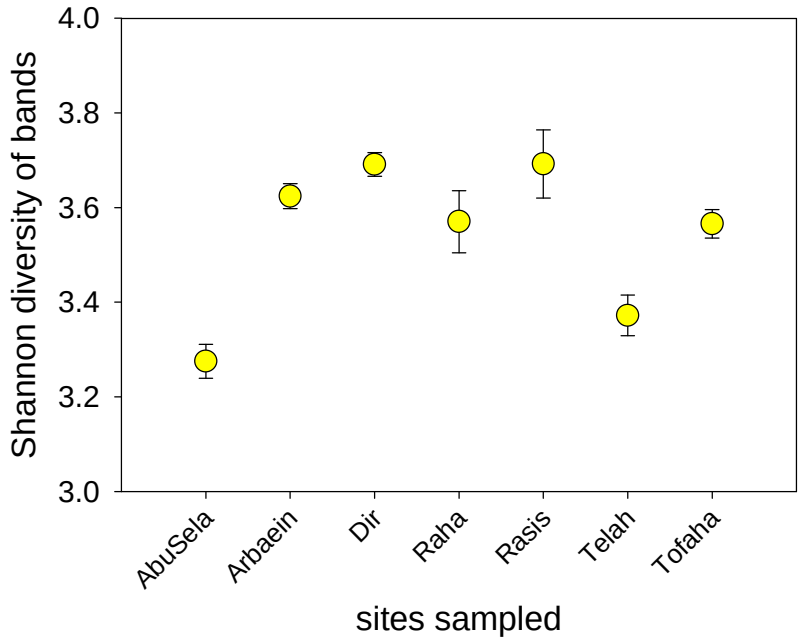

Fig 4. Plot of the average (± s.e.) band diversity for the seven sampled sites.

Wadi	N	bands	H	mean H
Arbaein	10	376	3.795	3.624
Tofaha	10	355	3.731	3.566
Dir	10	402	3.994	3.691
Rasis	3	121	4.026	3.692
Raha	4	143	4.080	3.570
Telah	10	294	3.735	3.214
AbuSela	4	106	3.499	3.275
		mean	3.837	3.549
total	51	1797	4.185	± 0.047

Table 1: Summary of RAPD band diversity among the sampled sites. N=number of individuals sampled; bands = total number of bands registered; H = Shannon-Wiener diversity of bands (data pooled across individuals); mean H = band diversity averaged for the individuals sampled. The s.e. of the total diversity results from a jackknife procedure.

Discussion

Anthophora pauperata is an important early-spring pollinator in the mountains of South Sinai, and in particular for *Alkanna orientalis*. The bees are more or less obliged to forage from this plant because it is the only really common plant flowering at this time: female bees can collect both nectar and pollen (by buzzing: Semida 1994; Stone *et al.* 1999).

Here we used RAPD markers for an initial evaluation of the population genetic structure in *A. pauperata*. A considerable amount of genetic variation was detected by the five oligonucleotide primers, mostly within-sites but also a significant level of between-site variation such that virtually all sites differed from every other site. The only two sites where bees were not detected as genetically different were also those with the lowest sample sizes (El Rasis and El Raha), but there are good *a priori* reasons to suspect that these two sites might show close genetic similarity since they are relatively close (about 1.5 km), with a broad open area between them without geographical barriers. Low genetic variability is not always evident in small samples (Moraes *et al.* 2005; Honnay *et al.* 2007).

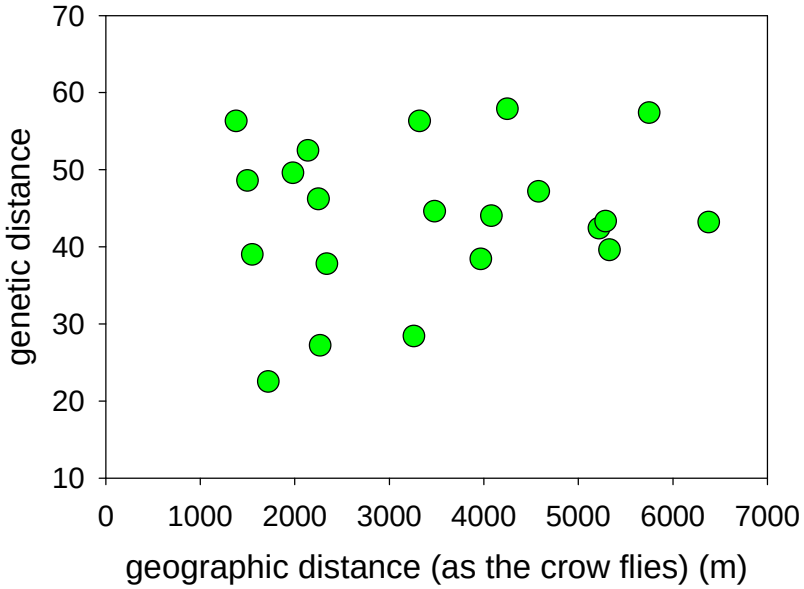

Fig 5 A scatterplot of genetic dissimilarities and geographic distances (measured as the crow flies from a satellite image): there is no significant relationship as assessed by a Mantel test.

Female *A. pauperata* may mate once, or perhaps several times, once for each egg laid (Gilbert 1999); if the latter, then potentially this could create high gene flow within populations, homogenizing their genetic makeup. However, what we see is high genetic diversity among individuals within sites, more consistent with single matings. The significant differences in the genetic structure among sites is unrelated to distance, but the distances among sites are very small relative to other studies of genetic substructuring of populations. Thus the results indicate some degree of genetic isolation for the *Anthophora* bees of these sites, with perhaps limited gene flow.

One possible cause of limited gene flow might be the foraging areas of males and females. Three kinds of behaviour are exhibited by *A. pauperata* males (Willmer *et al.* 1994; Stone *et al.* 1999; Gilbert 1999): some are territorial, flying around a few *Alkanna* plants until they die of exhaustion; others adopt a sit-and-wait strategy, minimizing energy expenditure; and a few males appear to be 'floaters', moving much more widely. Females appear to forage around their nests over an area that is a bit larger than a male's territory, but which overlaps with those of several males. After they have dug, provisioned and oviposited in the 3-5 cells of their nest, the female seals it with small stones (Semida 2000) and flies off, presumably to dig another. The bits of evidence we need are the mating success of the three types of males, and the typical distance a female moves to start another nest. These are crucial elements of an explanation of the genetic structure of the population. It is certainly possible that virtually all matings are achieved by territorial males, and that females move only very short distances: both would contribute to the limited gene flow suggested from the genetic evidence presented here.

A further component limiting gene flow is the fact that the flight season of *A. pauperata* occurs in late March and early April, when air temperatures are very low. The bee is a superb thermoregulator (Stone *et al.* 1999), but inevitably such abilities come at an energetic cost, making unlikely any long flights up and over the high mountainous barriers lining each wadi, some 5-600 m higher than the wadi bed. The altitude of the mountains of St Katherine and the highly dissected and fragmented nature of the wadi habitats must play an

important role in genetic differentiation because it increases the distances bees need to fly to reach neighbouring populations.

An additional possibility to explain significant genetic variability among sites is the fact that *A. pauperata* is a relatively specialized bee in its foraging behaviour. Zayed *et al.* (2005) suggested that specialist bees had significantly less variation within but more more genetic differentiation among their populations than generalists. The reason is that specialists persist in more isolated populations than generalists.

The evidence for genetic differentiation in this solitary bee fits with other lines of evidence about significant differences among wadis in the environment of the St Katherine Protectorate. There are big differences in plant communities (Zalat *et al.* 2001; Guenther *et al.* 2005), including morphological and genetic differences in the bee's main food plant *Alkanna orientalis* (Gilbert *et al.* 1996; Wolff *et al.* 1997; El-Akkad *et al.*, in press); there are consistent differences in pollinator communites (Gilbert *et al.*, unpublished data); and there are amazing differences in the metazoan (Behnke *et al.* 2000, 2004) and intestinal protozoan (Bajer *et al.* 2006) parasite communities of small rodents, which has affected their behaviour (Barnard *et al.* 2003). Why there are such huge differences across such small distances is a key issue for which answers are required.

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الملخص العربى

التباين الوراثى لعشائر النحل البرى المتوطن لمصر من النوع أنثوفرا بيبوراتا فى محمية سانت كاترين

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4- قسم علوم الحياة والبيئة – جامعة نوتنجهام – المملكة المتحدة

من خلال هذا البحث تم دراسة التركيب والتباين الوراثى لأفراد وعشائر النحل البري الإنفرادي "آنثوفورا بيبوراتا" لمعرفة تأثير العزل الجغرافى الطبيعى فى محمية سانت كاترين ومدى تأثير هذا العزل على التركيب الوراثى لعشائر النحل البرى فى المنطقة . تم إختيار الوديان التالية: وادى الأربعين، وادى الدير، منطقة الرصيص، وادى التفاحة، سهل الراحة، منطقة ابو سيلة، وادى إطلاح، تم تجميع النحل من كل منطقة وعزل الحمض النووى لكل الأفراد، وبيان التركيب الجينى لها بإستخدام تقنية الرابد "RAPD" وذلك لبيان مدى التطابق او الاختلاف فى التركيب الوراثى للافراد داخل نفس العشيرة فى الوادى الواحد، وأيضا بين العشائر المختلفة فى الوديان المختلفة. ولقد أثبتت النتائج:-

1- قدرة تقنية الرابد في تحديد درجات التطابق والاختلافات و التنوع الوراثي على مستوى الأفراد والعشائر لهذا النوع من النحل البرى

2- أن التركيب الوراثى لأفراد النوع داخل الوادى الواحد تتشابه مع بعضها البعض ولا توجد إختلافات وراثية معنوية (طبقا للتحاليل الإحصائية) داخل افراد الوادى الواحد

3- أوضح التحليل الإحصائى وجود اختلاف معنوى واضح فى التركيب الوراثى لأفراد العشائر فى الوديان المختلفة بمعنى أن أفراد كل منطقة تتشابه بصورة أو بأخرى مع مثيلاتها فى نفس الوادى ولكن يختلف التركيب الوراثى للعشيرة بشكل عام عن العشيرة الاخرى فى الوادى الآخر مما يعنى وجود وجود عزل وراثي بين عشائر ***(Anthophora pauperata)*** نتيجة للاختلافات الموجودة في القطع الجينية **(DNA fragments)** بين الوديان المختلفة.

4- أوضحت الدراسة أن عشائر النحل فى الوديان : إطلاح – الأربعين – التفاحة – أبو سيلة – الدير تختلف إختلافاً معنوياً عن بعضها البعض من حيث التركيب الوراثى على العكس من عشائر النحل فى منطقة الرصيص وسهل الراحة التى تقاربت تراكيبها الوراثية بصورة كبيرة حيث أن المنطقيتن مفتوحتين ولا توجد عوازل طبيعية تعوق أو تمنع طيران النحل بينهما مما بسمح بإنسياب الجينات الوراثية من خلال التزاوج بين أفراد المنطقيتن