Molecular insights into an insect-plant interaction

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

[وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (68) ثُمَّ كُلِي مِنْ كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِنْ بُطُونِهَا شَرَابٌ مُخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِلنَّاسِ إِنَّ فِي ذَٰلِكَ لَآيَةً لِقَوْمٍ يَتَفَكَّرُونَ (69)] {النحل: 68-69}



In the name of God, the most Gracious, the most Merciful

[And your Lord inspired the bee: “Set up hives in the mountains, in the trees, and in what they construct’’ (68) Then feed on every kind of fruits, and go along the pathways of your Lord, with precision. From their bellies emerges a drink of diverse colours, containing healing for the people. Surely in this is a sign for people who give thought (69)] {16. The Bee: 68-69}

الملخص باللغة العربية

يقوم هذا المشروع بتقييم الدراسة البيئية (الإيكولوجية) والجينية (الجزيئية) في تفاعل النبات مع الحشرات، باستخدام نبات الكانا أورينتاليس أو مايسمى بالعُسَّيْلا وهو من الفصيلة الحُمْحُمِيَّة وملقحه حشرة الأنثوفورا بوبيراتا وهو من رتبة غشائية الأجنحة - فصيلة أنتوفوريدى كنموذج في الشرق الأوسط. حيث يتزامن وقت الإزهار للنبات مع وقت ظهور الملقح الرئيسي للنبات. كما سيساهم فهم العلاقة بين النبات وملقحه على التوصل إلى توصيات أفضل بشأن استراتيجيات الحفظ. نتائجنا من وجهة نظر الدراسة البيئية تبين لنا أن حشرة الأنثورا في الجزء الشمالي من المرتفعات الغربية من المملكة العربية السعودية تحديداً في جبل اللوز كانت من الزوار الأكثر شيوعاً لكل من حبوب اللقاح والرحيق لنبات الكانا، وكان أيضا الملقح الفريد القادر على نقل حبوب اللقاح من نبات إلى آخر.في حين تقوم الإناث بجمع الرحيق وحبوب اللقاح في وقت مبكر وفي وقت متأخر من اليوم، إلا أن الذكور تعمل على الدفاع عن تلك الأراضي بين هذه الأوقات. كما تبين لنا في هذه الدراسة أن الإناث قادرات على السفر لمسافات طويلة بغض النظر عن العوائق الطبيعية والحواجز الجبلية العالية، وبالتالي فهي قادرة على نقل الجينات (المسؤلة عن الصفات الوراثية) بين الوديان المتباعدة والغير متصلة.

فمن الناحية الجزيئية للنبات، قمنا بالتحقيق في الاختلافات الجينية ضمن وبين المجموعات السكانية لنبات الكانا أورينتاليس على نطاقات مكانية مختلفة عبر توزيعها الجغرافي في مناطق الشرق الأوسط وشرق البحر الأبيض المتوسط. وقد استخدمنا الحمض النووي الريبوسومي (nrDNA) الموروث بشكل ثنائي من قبل الأبوين (منطقة ITS)، وكذلك الحمض النووي للبلاستيدات الخضراء (cpDNA) المورث من قبل الأمهات فقط (منطقة trnH-psbA)، لفهم معدل التدفق الجيني لحبوب اللقاح مقابل البذور. وقد لوحظت أدلة على الهيكلة المكانية على النطاق الجغرافي الكبير، والتي قد تكون نتيجة لسلسلة تاريخية من التوسعات والانقباضات. وعلى الرغم من وجود مثل هذه الأنماط الواسعة النطاق، فقد تم الكشف عن أدلة على عدم التجانس المكاني على النطاقات الصغيرة، مما قد يصف وجود حوادث محلية أكثر حداثة مثل الإقراض السكاني وإعادة الاستعمار من المناطق المجاورة، أو تقلبات في حجم السكان ترتبط بالانجراف الوراثي العشوائي عندما يكون حجم المجموعة السكانية صغير.

أما من الناحية الجزيئية للملقح، فقد قمنا بتحديد الاختلاف الجيني ضمن وبين المجموعتين السكانيتين الوحيدتين لحشرة الانثوفورا في جبل اللوز في شمال غرب المملكة العربية السعودية وجنوب سيناء، مصر. في هذه الدراسة قمنا بترجمة جزء من الحمض النووي للميتوكوندريا وعشرة مناطق شديدة التباين من الميكروستالايت. وقد

أكدت الأدلة على وجود تمايز وراثي كبير على النطاق الجغرافي الكبير بين المجموعتين السكانيتين. وعلى الرغم من وجود مثل هذه الأدلة الواضحة على النطاق الجغرافي الواسع، إلا أن هناك دلائل على حدوث تدفق جيني كبير داخل المجموعة السكانية الواحدة على النطاقات الجغرافية الصغيرة، مثل الأودية.

وبشكل عام، ومن نتائج هذه الدراسة، ينبغي أن تمنح المناطق ذات المستوى العالي من الاختلافات الجينية الإهتمام الأكبر، ومن ثم يتبعها المجموعات السكانية التي تقع تحت التهديد الشديد بسبب سوء استخدام الإنسان للأراضي. لذلك، بالنسبة لنبات الكانا، فيجب أن تمنح المجموعات السكانية التي تقع في شرق البحر الأبيض المتوسط الأولوية الكبرى في عملية الحفظ، ومن ثم تليها المجموعات السكانية في جنوب غرب المملكة العربية السعودية. أما بالنسبة لحشرة الأنثوفورا، فإن المجموعة السكانية التي تقع في جنوب سيناء يجب أن تحظى بالأولوية الكبرى في عملية الحفظ، ومن ثم تليها المجموعة السكانية في الشمال الغربي من المملكة العربية السعودية.

Abstract

This project evaluates the phylogeographic patterns of an insect-plant interaction, using ecological and molecular data for the model system of *Alkanna orientalis* (Boraginaceae) and its solitary bee pollinator *Anthophora pauperata* (Anthophoridae) in the Middle East. Understanding the relationship between the plant and its pollinator will help with recommendations for conservation strategies.

The flowering time of the plant coincided with the appearance of its main pollinator. In the northern part of the western highlands of Saudi Arabia, *A. pauperata* was the most common visitor for both pollen and nectar, and was also an efficient pollinator, transferring pollen to the stigmas. The females foraged for nectar and pollen early and late in the day, while the males performed territory defence in between these times. Females were shown to be able to travel long distances regardless of the harsh landscape and high mountain barriers, and hence can facilitate genetic exchange of both bee and plant amongst wadis.

From a molecular point of view, I investigated genetic variation within and amongst populations of *Alkanna orientalis* at different spatial scales across the eastern Mediterranean and Middle East regions. I used biparentally inherited, nuclear ribosomal DNA (the ITS region) and maternally inherited, chloroplast DNA (the trnH-psbA region) to understand the rate of gene flow of pollen *vs.* seeds. The results showed evidence of spatial structure over a large geographical scale, which might be the consequence of historical series of expansions and constrictions. In addition to these large scale patterns, there was evidence of spatial heterogeneity at smaller scales, which may describe more recent, localised events such as population extinction and recolonization

from adjacent territories, or fluctuations in population size associated with random genetic drift when population size is small.

For the pollinator, *Anthophora pauperata*, I describe the genetic variation within and between the only two known populations, at Jabal El-Loz in northwest Saudi Arabia and the high massif of South Sinai in Egypt. For this I sequenced a protein-coding region of mitochondrial DNA, and genotyped ten hyper-variable microsatellite loci. The results showed evidence of significant genetic differentiation across the large geographical scale between the two populations, coupled with significant gene flow within populations over smaller geographical scales, such as between wadis.

One of the outcomes of this study is to suggest that some populations should be given higher priority in terms of conservation. This prioritisation is based on elements, such as the level of genetic diversity within each population, the distribution of unique genotypes, evidence that populations have been recently exposed to bottlenecks, populations that are potentially glacial refugia, and populations under extreme threat because of human activities.

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My all praise and gratitude are due to almighty god (Allah), the most gracious the most merciful, who made this project easy. I ask him with all hope to accept this work and make it purely to meet his satisfactions.

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List of Contents

الملخص باللغة العربية III

Abstract V

Acknowledgements VII

Chapter 1: General introductio 1

1.1 Plant-insect interactions 3

1.2 The ecological and genetic effects of dispers 8

1.3 The geographic mosaic 12

1.4 Thesis outline 15

1.5 References 17

Chapter 2: Flower visitors to *Alkanna orientalis* (Boraginaceae) in Saudi Arabia and their pollination effectiveness 23

2.1 Abstract 24

2.2 Introductio 25

2.3 Materials & Methods 27

2.4 Results 31

2.5 Discussion 57

2.6 Interesting observatic 64

2.7 Acknowledgements 65

2.8 References 66

Chapter 3: Daily pattern of territoriality in an anthophorid bee: a case study from Jabal El-Loz, northwestern part of Saudi Arabia 75

3.1 Abstract 76

3.2 Introductio 77

3.3 Materials & methods 79

3.4 Results 83

3.5 Discussion 98

3.6 Acknowledgements 103

3.7 References 104

Chapter 4: Phylogenetic and phylogeographic analysis of *Alkanna orientalis* (Boraginaceae) in the Middle East 110

4.1 Abstract 111

4.2 Introductio 112
4.3 Materials and methods 113
4.3.1 Study area 113
4.3.2 Study system 114
4.3.3 Sampling 114
4.3.4 DNA extraction and purificatior 115
4.3.5 Genetic marke 115
4.4 Results 117
4.4.1 nrDNA: ITS 117
4.4.2 cpDNA: trnH-psbA 118
4.5 Discussion 133
4.6 Acknowledgements 136
4.7 References 137
Chapter 5: A population and phylogenetic analysis of an Anthophorid bee: *Anthophora pauperata* (Boraginaceae) in Saudi Arabia and Sinai 142
5.1 Abstract 143
5.2 Introductio 144
5.3 Material and methods 146
5.3.1 Study area 146
5.3.2 Study system 147
5.3.3 Sampling 147
5.3.4 DNA extraction and purificati 148
5.3.5 Genetic marke 148
5.3.5.1 Mitochondrial DNA 148
5.3.5.2 Microsatellites 149
5.3.6 Statistical analy 150
5.3.6.1 Molecular markers 150
5.3.6.2 Population structur 151
5.4 Results 152
5.4.1 mtDNA 152
5.4.2 Microsatellites 153
5.5 Discussion 173
5.6 Acknowledgements 177
5.7 References 177

Chapter 6: General discussion 187

6.1 From an ecological point of view 188

6.1.1 Is Anthophora pauperata the main pollinator of *Alkanna orientalis*? 188

6.1.2 *Anthophora pauperata* movements..... 189

6.1.3 The foraging and mating patterns *Anthophora pauperata*..... 190

6.2 From a molecular point of view 193

6.2.1 In the plant 193

6.2.2 In the bee..... 194

6.3 Are the ecological findings consistent with the molecular findings in the north western part of Saudi Arabia?..... 195

6.4 Conclusion 196

6.5 References..... 198

Supplementary.....202

List of Tables

Table 2.1: Studied plots with elevations and location a -Loz (Madiyan Mountains range, Tabuk region, NW Saudi Arabia). 35

Table 2.2: Visitor taxa seen on the flowers and plants of *Alkanna orientalis* at Jabal El-Loz in March-April 2015. Photographs of some of these taxa are shown in Figure S2. 36

Table 2.3: Overall numbers of the visitors to the flowers of *Alkanna orientalis* in five plots in Jabal El-Loz, Saudi Arabia. The left side of the table shows the dates of observations and the plant density for each 100x100 m plot that was studied. middle part shows the number of observed plants with the total number of flowers at the female stage, the number of individual visitors and the number of visited flowers during the observational periods. The right side shows the species of flower visitors, the number of observed individuals and the total number of visited flowers. 37

Table 2.4: ANCOVA table for the effect of three predictors on the number of visits to flowers of *Alkanna orientalis*; one predictor is a covariate (the number of flowers at the female stage), and the others are factors. 39

Table 2.5: ANOVA of the number of pollen grains delivered to the stigma *Alkanna orientalis* as predicted by wadi and pollination treatments. The test was done by sequential deletion, with Poisson errors, tested by Chi-squared. 40

Table 3.1: ANOVA with binomial errors of the proportion of m *Anthophora pauperata* bees remaining at the same site through time, as predicted by site, sex, and week. The test was done by sequential deletion, tested by chi-squared. 87

Table 4.1: Primer sequences of the nuclear and chloroplast DNA. 121

Table 4.2: Summary of SNPs that generate different haplotypes of *Alkanna orientalis* based on nuclear ribosomal DNA (ITS region) across eight countries of the Middle East. 122

Table 4.3: Summary statistics for nuclear ribosomal DNA haplotypes (ITS region)..123

Table 4.4: Table indicating SNPs (shown in red) that generate the different haplotype: of *Alkanna orientalis* based on chloroplast DNA (trnH-psbA region). The two main

haplogroups subsequently identified in the network analysis are shown in green and yellow respectively. 124

Table 4.5: Summary statistics for chloroplast DNA haplotypes (trnH-psbA region). Saudi Arabian populations are shown individually and with populations from the two regions (northwestern and southwestern) grouped together. 125

Table 5.1: The selected microsatellite loci for *Anthophora pauperata* from related species. 156

Table 5.2: List of *Anthophora* populations genotyped. 157

Table 5.3: Primer sequences of the 10 microsatellites and fluorescent tag used. ...158

Table 5.4: SNPs that generate the different haplotypes of *Anthophora pauperata* based on mtDNA (cytochrome c oxidase subunit I & II and tRNA regions). The highlighted nucleotides in COI change amino acid from Phenylalanine in H1 (TTT) to Leucine in H2 and H3 (CTT). 159

Table 5.5: Genetic diversity and private haplotypes measured fc the two populations of *Anthophora pauperata* across three regions of mtDNA marker. 160

Table 5.6: Genetic diversity measures fc ten microsatellites loci and 78 *Anthophora* bees. 161

Table 5.7: Measures of significant differentiat $_{st}$ value) within and among populations for ten microsatellites loci and 7! *Anthophora* bees. 162

Table 5.8: Three tests for heterozygosity excess (Wilcoxon test, Standardized differences test & Sign test) under the two mutational methods in the two populations of *Anthophora pauperata*. 163

List of Figures

Figure 2.1: St Katherine in South Sinai (Egypt), where the only other study of *Alkanna* and *Anthophora* took place, and Jabal El-Loz, Tabuk region (north-west Saudi Arabia), where the present work occurred, separated by the Gulf of Aqaba. There is a distance of about 140 km between the study sites. 41

Figure 2.2: Study plots (red pegs) in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The white lines show the path of the wadis. The yellow pegs show plots that were established but unable to be used because of security considerations. 42

Figure 2.3: Habitus of *Alkanna orientalis*: (a) a typical plant with several hundred yellow flowers; (b) the flowers with their bracts covered with glandular hairs; (c) the red-coloured root; (d) the bilocular anthers, and single gynobasic style; (e) seeds forming after flower abscission. 43

Figure 2.4: World distribution o *Alkanna orientalis*. 44

Figure 2.5: Mean (± se) microclimatic conditions (relative humidity [%], temperat (°C), wind speed [km/hr]) for each hour of the day (GMT+2) for each plot, together with the overall mean values across plots at Jabal El-Loz. 45

Figure 2.6: The pattern of activity (no. of visitors per 0-minute observation) of all type visitors to flowers of *Alkanna orientalis* by local time of day (GMT+2) across five plot during March-April 2015. 46

Figure 2.7: The correlation between the number of flowers per pl *Alkanna orientalis* and the number of visits by insects to their flowers, from timed observations during March-April 2015. 47

Figure 2.8: Overall pattern (means ± se) of the foraging of male *Anthophora pauperata* during the day (GMT+2) across the five studied plots during March-April 2015 (N = nectar only; PN = collecting pollen & nectar 48

Figure 2.9: Female *Anthophora pauperata* digging nests during the middle of the day. (left) 13 March 2015 from wadi 1B, under Artemisia herl -alba; (right) 02 April 2015

from wadi 4A under *Alkanna orientalis*. Both were observed at Jabal El-Loz, Tabuk region (north-west Saudi Arabia).....49

Figure 2.10: Mean (± se) number of pollen grains deposited (A) among the three treatments of no visits, a single visit, and open visitation (many visits); and (B) among the three wadis used in the experiment.....50

Figure 2.11-A: Number of pollen grains deposited after a single visit by various taxa of visitors (n=90 flowers in total). 51

Figure 2.11-B: The number of pollen grains deposited after a single visit split by taxa and site.....52

Figure 2.12: (A) ANOVA with negative binomial of the differences among visitor ta in the total number of *Alkanna orientalis* flowers visited per 10-minute sample (means ± se). The differences among means are significant ($\chi^2 = 60.9$, df = 6, $p<<0.001$), female of *Anthophora pauperata* accounted for by the lower value of male ($Z = -2.7$, $P < 0.01$). The test was done by sequential deletion, tested by chi-squared. (B) Total number of visitors per 10-min sample.....53

Figure 2.13: Probability of being pollinated (proportion of seeds set) of the flowers o *Alkanna orientalis* against flower sequence, counting from the current flower (0) back down the flowering spike in the temporal sequence of flowering (hence equivalent to time in flower units), for each of the three plots (1A, 2A, 3A 54

Figure 2.14: The probability of pollination of each *Alkanna orientalis* flower increased in older flowers and then declined in very old flowers (± 95% confidence limits, obtained from the proportions, following Sokal et al. (1995 55

Figure 2.15: This figure shows the number of flowers per plant of *Alkanna orientalis*, and the number of visits per 10-min sample in each of the five sites at Jabal El-Loz (Saudi Arabia), ordered by the date of observation. It is obvious that the number of visits is decreasing through time. 56

Figure 3.1: Study plots in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The red pegs show the plots studied for male territorial behaviour. Both the yellow and red pegs show plots used for studying bee movements. The white lines

show the path of the wadi beds. The highest elevation was site 1A, and the lowest site 7A. 88

Figure 3.2: Map from site 1A at Jabal Al-Loz on March 2018, showing the patrolling routes of the observed male *Anthophora pauperata* (#40) and his neighbour (#42). Male #40 used three types of loops around six *Alkanna orientalis* plants of different sizes containing 538 flowers in total. The three patches of his neighbour #42 and his movements are also shown. Circles represent plants, labelled with the total number of open flowers. 89

Figure 3.3: Map from site 2B at Jabal Al-Loz on March 2018, showing the patrolling routes of a male *Anthophora pauperata* using two types of loops around 9 *Alkanna orientalis* plants of different sizes containing 793 flowers in total. Circles represent plants, labelled with the number of total open flowers. 90

Figure 3.4: Map from site 3A at Jabal Al-Loz on March 2018 is showing the patrolling routes of a particular male *Anthophora pauperata* showing different types of loops around 24 *Alkanna orientalis* plants of different sizes containing 1320 flowers in total. In the early morning, the patrolling male showed three types of loops in a first set of 11 plants (1) (black routes). Later on, he added a second set of six plants and showed two types of loops around them (2) (pink routes). Even later on the second day, he added a third set of five plants and showed two types of loops around them (3) (blue routes). Before the end of the second day of observation, he added a fourth set of two large plants to his territory (4) (red routes). Circles represent plants, labelled with the number of total open flowers. 91

Figure 3.5: (A) Mean (± se) number of male-female interactions of *Anthophora pauperata* per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are significant ($F_{8, 80}$ = 3.221, P = 0.003). (B) Mean (± se) number of male-female interactions of *Anthophora pauperata* per 10-min observation period among three wadis. The differences among means are significant ($F_{2, 86}$ = 3.4, P = 0.03), all accounted for by the higher value of wadi 2B. 92

Figure 3.6: (A) Mean (± se) number of male-male interactions of *Anthophora pauperata* per 10-min observation period along the day local time across three different sites during March-April 2015. Despite the appearance of especially 0800 vs 0900, overall the differences among means are not significant ($F_{8, 80}$ = 1.344, P = 0.23). (B) Mean (± se) number of male-male interactions of *Anthophora pauperata* per 10-min observation period among three wadis. The differences among means are significant ($F_{2, 86}$ = 4.898, P = 0.009), all accounted for by the higher value of wadi 1A. 93

Figure 3.7: (A) Mean (± se) number of *Anthophora pauperata* male sittings on the ground per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are highly significant ($F_{8, 217}$ = 25.34, P << 0.001). (B) Mean (± se) number of *Anthophora pauperata* male sittings on the ground per 10-min observation period among three wadis. The differences among means are highly significant ($F_{2, 223}$ = 23.98, P << 0.001), all accounted for by the lower value of wadi 3A. 94

Figure 3.8: (A) Mean (± se) number of visits of *Anthophora pauperata* male to flowers of *Alkanna orientalis* per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are highly significant ($F_{8, 170}$ = 44.82, P << 0.001). (B) Mean (± se) number of visits of male *Anthophora pauperata* to different clump sizes of *Alkanna orientalis* flowers per 10-min observation period throughout the day across three different sites during March-April 2015. The differences among means are significant ($F_{2, 174}$ = 8.54, P = 0.0002). 95

Figure 3.9: The movements of female *Anthophora pauperata* between sites at Jabal El-Loz in the northern part of the western highlands of Saudi Arabia during March-April 2015. 96

Figure 3.10: Mean (± s.e.) percentage of marked male and female *Anthophora pauperata* remaining in the same site through time (weeks) across twelve different sites during March-April 2015. 97

Figure 4.1: Map showing eight countries where *Alkanna orientalis* were sampled or obtained for this study. The circles represent the population genetic structure based on nuclear ribosomal DNA (ITS region) across the eight countries. 126

Figure 4.2: Nuclear ribosomal DNA (ITS region) haplogroups in *Alkanna orientalis*. Small red squares represent nodes, which correspond with inferred missing haplotypes or homoplasies. Branch lengths are proportional to the number of mutations. The haplotypes are coloured regarding to their nine geographical groups. The outgroup species are *Alkanna tinctoria* and *Alkanna sieberi*. Circle size is proportion haplotype frequency. 127

Figure 4.3: Genetic relationship bet *Alkanna orientalis* based on nuclear ribosomal DNA in ITS region over Middle East range. The related outgroup species were selected from GenBank based on BLAST searching for closest sequence matches. 128

Figure 4.4: Map showing eight countries where *Alkanna orientalis* were sampled or obtained for this study. Each circle represents the origins of samples with the number of individuals has been analysed based on chloroplast DNA (trnH-psbA region). 129

Figure 4.5: Chloroplast DNA (trnH-psbA region) haplogroups in the Middle East range identified from 180 specimens o *Alkanna orientalis* and an outgroup species (*Alkanna tinctoria*). Small red squares represent nodes, which correspond with missing haplotypes or homoplasies. Branch lengths are proportional to the number of mutations. The haplotypes are coloured regarding to their thirteen geographical groups. Circle size is proportion haplotype frequency. 130

Figure 4.6: Neighbour-joining dendrogram of the individual *Alkanna orientalis* based on chloroplast DNA in trnH-psbA region. Individuals are from different wadis at St Katherine, south Sinai. Each colour represents different wadi. 131

Figure 4.7: Neighbour-joining dendrogram of the individual *Alkanna orientalis* based on chloroplast DNA in trnH-psbA region. Individuals are from different wadis at Jabal Al-Loz, northwestern Saudi Arabia. Each colour represents different wadi. 132

Figure 5.1: Study plots in wadis on the South of flank of Sinai, Egypt. The highest and lowest elevations from the sea level occurs in wadi Arbaein and wadi Abu Seila respectively. 164

Figure 5.2: Study plots in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The highest and lowest elevations from the sea level occurs in site 1 and site 4A respectivel 165

Figure 5.3: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (encoding region of cytochrome c oxidase subunit I). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Two haplotypes belong to Jabal Al-Loz and one to St Katherine. Node numbers refer to Bootstrap support (1000 replicates). 166

Figure 5.4: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (encoding region of cytochrome c oxidase subunit II). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). One haplotype belongs to Jabal Al-Loz and five to St Katherine. Node number refer to Bootstrap support (1000 replicates). 167

Figure 5.5: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (tRNA non-coding region in-between cytochrome c oxidase subunits I and II). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Two haplotypes perfectly differentiate each region. Node number refer to Bootstrap support (1000 replicates 168

Figure 5.6: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (a combination of all three regions; COI, tRNA, and COII). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Seven haplotypes differentiate the two populations. Node numbers refer to Bootstrap support (1000 replicates). 169

Figure 5.7: Allele frequencies per microsatellite locus across the two populations *Anthophora pauperata*. Ns: northwestern Saudi Arabia population; Si: South Sinai population. 170

Figure 5.8: Graph representing the optimal clustering m 2 to 7), from STRUCTURE HARVESTER analysis.....171

Figure 5.9: Individual assignment based on the Bayesian method under an admixture model obtained by STRUCTURE. 78 *Anthophora* bees from two populations were analysed at K = 2 and = 3 (K is the assumed number of popula subpopulation is represented by a single line and their colours correspond to the relative proportion of the inferred genome corresponding to a specific genetic cluster. The two main populations are separated by the long black line. Ns: northwestern Saudi Arabia population; Si: south Sinai population. Numbers in x axis represent subpopulation sites.....172

Chapter 1: General introduction

Loss of biodiversity is a key issue in the world that threatens human life and well-being: it is being intensely researched by scientists today. One of the most important factors involved in this deflection of natural ecosystems is human use of the landscape for economic purposes. Humans destructively impact biodiversity and can cause the extinction of species worldwide (Baguette *et al.*, 2013). This is not new: Burney and Flannery (2005) discuss the late prehistoric period when humans spread across the Earth and affected previously uninhabited areas. Contact with humans resulted in the mass extinction of the megafauna, together with losses of smaller animals and plants within a few centuries.

Today one of the main factors causing extinctions beside habitat destruction is agricultural practices, such as the excessive use of pesticides and fertilizers that can lead to destructive effects such as the loss of pollinators essential to most plant reproduction and crop yield, specially angiosperm (Ollerton *et al*., 2011). The annual estimation of the global economic value of pollination ranges between $112 and $200 billion (Kremen *et al.*, 2007) and the cost of losing pollinators in Egypt was estimated at about $2.4 billio in 2004 (Brading *et al.*, 2009). Evidence of pollinator extinctions is widespread; for example, in a study of the temperate forest in Illinois, Burkle *et al.* (2013) compared changes over 120 years in plant-pollinator interactions, finding clear decreases through time of functional groups and the interaction network; half of the bee species have died out because locally extinct. Deterioration of natural ecosystems is the main driver of biodiversity loss (Baguette *et al.*, 2013), with habitat loss and fragmentation leading to population extinction. Other important factors are pollution, climate change, overexploitation, the presence of invasive alien species and the loss of intraspecific genetic diversity (Manel *et al.*, 2013), the last of which is proposed to be a key driver of

the extinction of entire populations. Understanding from different disciplines including landscape genetics can improve the way in which we deal with the connected problems of fragmentation, connectivity between elements of a metapopulation, gene flow and adaptation (Manel *et al.*, 2013). Such understanding would materially affect conservation strategies, specifically by determining which populations are the important ones to conserve first (Schemske *et al*., 1994; Arponen, 2012; Winter *et al*., 2013).

1.1 Plant-insect interactions

Great attention has been paid to the relationships between plants and insects, because many ecologists and evolutionary biologists consider that these interactions are crucial to understanding plant diversification. The interaction was traditionally regarded as mutualistic, with both elements interacting to mutual benefit, but in fact mutualism may have no direct or significant impact (Boucher, 1985), and modern thinking regards the interaction as mutual exploitation rather than helping each other because there are many instances where the benefits to the plant from its insect visitor are absent because it bypasses the stigma and anthers and pollination does not occur (Bronstein, 2001). There are a number of factors (Bronstein *et al.*, 2006) that explain why such mutual exploitation is critical in understanding plant evolution. First, plant reproduction is associated with the need to attract insect mutualists. Second, research on plant-insect mutualisms promotes better understanding of the function of a diverse suite of traits and mating systems of plants. Third, plant-insect mutualism is associated with patterns of evolutionary diversification. A final factor is its significance to the area of conservation. Bronstein *et al*. (2006) emphasise the three most prominent forms of plant-insect mutualism: pollination, protection, and seed dispersal.

Pollination is simply defined as the transfer of pollen grains from the anther of one individual plant to the stigma of another in order to fertilize the ovules. Most angiosperms need animal pollinators for this transfer of pollen, while animal pollinators rely on plants for nutrient sources, heat and oils: although mutualistic, the relationship therefore entails mostly asymmetric benefits (Willmer, 2011). Flowers are commonly hermaphrodite, with both male and female functions, and play a significant role in regulating the equilibrium between inbreeding and outbreeding which affects the genetic structure and eventually the evolutionary trajectory of the plant (Willmer, 2011). Since the plant cannot move, if it requires cross-pollination it needs a third party to transfer its pollen - either the wind, water or visiting animals: the last named is the most common method of pollination. The dynamics of pollination probably constitutes a potent force stimulating speciation in plants (Geber *et al.*, 2006; Willmer, 2011). Via its role in plant reproduction, the base for nutrient cycles worldwide, pollination is therefore essential for keeping the planet's ecosystems healthy, and also in securing the human food supply. There are more than 130,000 species of animal regularly visi flowering plants; most are Hymenoptera, largely bees (Apidae) (Willmer, 2011).

Most flower visitors seek the sugar of nectar, but many also collect the pollen itself, typically as a source of protein either for themselves or for their offspring. As well as attracting pollinators, flowers also provide a suitable protected warm microclimate for small animals for shelter, oviposition, mating, predation and thermoregulation. Plant traits important in attracting visitors include flower density, number, height or spatial patterns and even more traits at the level of the flower: shape, colour, scent, the volume of reward and period of flowering. For many species, visitors are ultimately required to

guarantee outcrossing, rather than selfing which leads to different predicted patterns of evolutionary change (Willmer, 2011).

Pollen was being consumed by insects during their early evolution, as shown by the presence of preserved gymnosperm pollen in fossil insect guts, but subsequently insects evolved long nectar-sucking mouthparts. The interactions between insects and flowering plants show parallel changes, and flowers with particular combinations of floral traits are visited more often by pollinators. This is an indirect kind of sexual selection, and shows that the diversity of angiosperms could have been brought about by biotic pollination. Diversification among the families and genera of plants is believed to have been influenced by shifts among functional groups of pollinators: bees, birds, moths, and other insects. Many ecologists have suggested that these shifts could be predisposed by changes in flower morphology, whose physical structure apparent to insects significantly affects how enticing they are (e.g. bilateral symmetry). Diversification of the bees along with the plants they pollinate may also contribute to shifts in pollen use (Bronstein *et al.*, 2006), and particular groups of bees that are rich in species usually include pollen specialists.

In one recent study, Anderson *et al.* (2014) investigated variation among populations in the length of the corolla of *Tritoniopsis revoluta* to understand how this flower evolved. They asked whether this type of evolution is associated with pollinator proboscis lengths, and whether corolla variants occur because of different pollinators. They tested whether distantly related populations with the same pollinator have similar floral morphology, and whether such evolution in floral morphology is directional. They found a positive linear relationship linking floral tube length with pollinator mouthpart length, and proposed that plants are evolutionarily specialized at the population level

rather than the species level. The evolution of *T. revoluta* flowers can occur in any direction to adapt to the shape of the local pollinators. Thus morphological divergence in flowers is a consequence of the geographical mosaics of pollinator morphology, irrespective of the distance between populations. A later study (Johnson *et al.*, 2017) supports the same findings, showing that hawkmoth pollinators have a binary or multi-modal distribution of proboscis length that is reflected in the distribution of corolla tube lengths. Greater corolla length means a greater degree of pollinator specialization to hawkmoths with long mouthparts, while the opposite is true of the pollinators, since hawkmoths with shorter mouthparts are more specialized in their foraging behaviour than those with longer mouthparts.

It is also advantageous to combine pollinator behaviour with the study of pollination and gene dispersal to understand how pollinators mediate the gene flow of the plants they visit. Cranmer *et al.* (2012) studied the flight behaviour of bumblebees in hedgerows, and also in artificial landscapes including analogous linear features, to determine how flight movements respond to such structures. They then followed this up with sentinel plants of *Salvia pratensis* to look at the consequences for pollen receipt and seed set. Flight orientation was affected in both natural and artificial landscapes, with clear consequences for plant reproductive success. Patches with more connecting corridors had greater pollinator activity, received more pollen and set more seeds. This study demonstrates clearly that landscape connectivity is important for pollinator movement and hence for the plant.

The second aspect of mutualism among plants and insects is protection: as an example, Bronstein *et al*. (2006) used the role of ants in protecting plants. The ant-plant association is very common, with up to 1/3 of all woody plants in particular ha

being involved. It shows a great range of forms, from facultative and generalized to obligate and highly specialized. In facultative generalized associations, the plant typically produces small volumes of extra-floral nectar from glands on leaves, flowers, or fruits, while ants keep other insects away from the plant, including herbivores. In this type of association, the ants manifest low levels of fidelity to particular plants. In rare specialized ant-plant associations, plants provide more valuable rewards such as provision of shelter and food, and some of the plant partners can benefit nutritionally by absorbing the waste deposited by ants within the domatia. Myrmecophytic ants manifest strong fidelity to individual plants. Although these appear to be mutualistic relationships, they involve plenty of deception and misuse (Bronstein *et al.*, 2006). Particular ant species attracted to a plant do not provide any benefits, and in fact can be detrimental, e.g. by interrupting successful pollination, reducing pollen viability or quantity and dissuading pollination visits (Ness, 2006).

Seed dispersal is a fundamental type of plant-animal mutualisms. The movement of plant seeds has a great impact on the spatial dynamics of their populations (Ouborg *et al.*, 1999), affecting gene movement, reproduction and persistence. In tropical lowland forests, nearly 98% of flowering plants depend on animals for either pollination or seed dispersal (Thompson, 1994). Commonly, the process of seed dispersal is facilitated by birds and mammals, either externally or internally through their guts, but ants are also significant contributors to seed dispersal, with about 17% of all angiosperm plants involved in myrmecochory, i.e. ant-mediated seed dispersal (Lengyel *et al.*, 2010). The availability of mutualistic ants is influenced by factors such as the timing of seed release and the ripening of the fruit (Bronstein *et al.*, 2006).

Plants attract vertebrate dispersers by offering fruit or berries and attract ants by offering food rewards such as nectar or elaiosomes, in order to disperse their seeds. Long-distance dispersal is facilitated by wind and vertebrates, while short-distance dispersal is facilitated by ants and various mechanical contrivances, for instance, ballistic methods of opening of the fruit. There are two characteristics used to describe the distribution of dispersed seeds: the relationship between seed density to distance from the source, plus the directionality in relation to the source (Willson *et al.*, 2000). Many further factors can play a role in altering the location of the peak, slope and shape of the spatial distribution of seed in specific cases. Some of these factors are plant-related, such as plant height, which may have strong environmental and genetic components, while others are environmental, such as the trend and strength of the wind, the behaviour of interacting animals, rainfall patterns and humidity. Still other factors are controlled by the interaction between genes and environment; for example, fruit and seed size, and the ease of abscission or dehiscence. Evolutionary changes in dispersal mode cause changes in appropriate aspects of seed morphology (Willson *et al.*, 2000). For example, a change from wind to bird dispersal in *Pinus* entails changes in cone structure and seed size (Benkman *et al.*, 1984). A change from bird to ant dispersal in *Acacia* may result in changes in colour and size of the nutritive skin attached to the seed (O'dowd *et al.*, 1986).

1.2 The ecological and genetic effects of dispersal

Dispersal has been simply defined (Matthysen, 201 as movement of an individual from its natal location to where it breeds. This can occur prior to breeding, there can be movement among sequential reproductive locations, or there can even be post-

breeding dispersal. It can be split into three stages: departure from the natal site, transfer through the landscape, and settlement in a new habitat (Baguette *et al.*, 2013; Matthysen, 201 .

There are thought to be some major and very different processes causing the evolution of dispersal. It is not simply a question of moving from an unfavourable site to more favourable one. The first is escaping from the risk of inbreeding and competition with kin, with the corollary of the possible costs of losing collaboration with kin, and of outbreeding (Baguette *et al.*, 2013; Matthysen, 20 . Forrest *et al.* (2011) studied optimal outcrossing distances using *Grevillea mucronulata* as a model organism, and found evidence of both inbreeding and outbreeding depression associated with genetic and geographic distances. *Grevillea mucronulata* showed the highest fitness in offspring produced from intermediate distances of pollen transfer, between 0.2 to 5 km, with outbreeding depression detected at greater distances. Second, dispersal results in the spreading of offspring into new and different environmental conditions. If these conditions are better, then expected fitness will increase, but if populations are locally adapted then movement could entail the cost of being maladapted, decreasing fitness. There is also the cost of motion itself, not just in energetic terms but also because moving over inappropriate habitat entails various mortality risks, such as from predators, or from starvation (Matthysen, 201 .

Successful dispersal is linked to physical landscape structure and the nature of the organism; in other words, it is linked to the medium through which the organism will disperse, as well as the fitness of the organism itself. Dispersal is the major cause of intra- and inter-population gene flow (Matthysen, 201 . The costs of dispersal increase as distance increases among fragmented habitats (Baguette *et al.*, 2013). Travelling of

individuals for long distances through inhospitable sections of landscape needs time and effort, and entails a high probability of failure. Such costs can be reduced either by reducing dispersal probability or enhancing dispersal efficiency by altering behavioural, physiological or morphological attributes. Dispersal is variable among individuals in timing and frequency, yet also within an individual lifetime due to ontogenetic changes or phenotypic plasticity. In some cases individuals may choose to leave an excellent quality habitat because of high levels of competition, or stay on in a poor quality habitat because of the costs of dispersal: thus the decision is not based solely upon habitat quality (Baguette *et al.*, 2013).

The connectivity of the landscape is a hard concept to make operational because it is influenced by many factors, not just the physical nature of the medium. There are also various ways in which it is calculated (Baguette *et al.*, 2013). This can be according to the spatial configuration of appropriate habitat patches, or by the functional connectivity, i.e. measurements of individuals moving through the landscape network of habitat patches. The costs that every landscape imposes on dispersing individuals have led to a new expression called landscape resistance: physical barriers or landscape elements will impede the movement of dispersing individuals that utilize particular modes of locomotion, leading to anisotropic dispersal. This in turn strongly affects estimates of structural connectivity. Even aerial dispersers are faced with differing atmospheric conditions, such as temperature and wind intensity and direction. Such anisotropic dispersal makes structural connectivity assessments less useful than they would otherwise be. The Euclidean distance (border to border, or core to core) is the easiest way to measure structural connectivity between populations (Baguette *et al.*,

2013), perhaps refined by organism behaviour (such as average dispersal ability) and the nature of the medium (e.g. resistance to movements).

There are also various indirect ways of estimating the functional connectivity of dispersal using gene flow via the calculation of dispersal kernels. It is important to measure gene flow as well as barriers toward gene flow using genetic tools, and then to look at the ways in which the landscape modifies gene flow using the techniques of landscape genetics (Baguette *et al.*, 2013). Such an approach will facilitate the assessment of landscape connectivity and its variability. If the costs of dispersal are very high then dispersing individuals might fail to reproduce once arrived in their new patch. Thus dispersal by itself does not automatically imply a lack of isolation and inbreeding, especially in small populations. Decreasing the costs of dispersal by improving connectivity via corridors might then have a dramatic influence on isolation (Alagador *et al*., 2012).

Rare long-distance seed dispersal is very important to study because of its effect on colonization (Ouborg *et al.*, 1999), but its low frequency makes the use of molecular markers vital. Markers differ based on their mode of inheritance and on the amount of variation which can either be dominant or co-dominant; they may also be either subject to genetic recombination or not. Seed dispersal can be measured effectively but indirectly by calculating genetic divergence among populations to estimate gene flow, finding a relationship with geographical distance, and splitting seed-mediated from pollen-mediated gene flow using nuclear *vs.* cytoplasmic molecular markers. Direct measurement involves parental assignment, which in turn requires highly variable molecular markers such as microsatellites. Genetic and ecological methods could complement each other in enabling understanding of plant gene flow (Ouborg *et al.*,

1999). The flow of genes also occurs via the pollination process as well, and different means of pollination entail different expectations of genetic differences (Wolff *et al.*, 1997): wind-pollinated species should manifest low levels, insect-pollinated species intermediate levels, and self-pollinating species the highest levels of genetic differences among populations.

1.3 The geographic mosaic

The current consensus is that one of the most significant factors driving evolution is the occurrence and extent of interspecific interactions (Darwin, 1859; Thompson, 1994). Such evolution in interacting species, i.e. coevolution, is a vital component that affects the worldwide structure of biodiversity. Older coevolutionary studies were mostly about pollinator-flower interactions and predator-prey-mimicry systems, trying to work out the way in which natural selection operated. Ehrlich *et al.*'s (1964) study of plant-butterfly coevolution caused an explosion of interest in the evolution of defences and counter-defences, the pattern of mutual alteration within local communities, and long-term fixed traits caused by the coevolutionary process.

A geographical approach to species interactions suggests estimating the patterns of covariation among populations of interacting species in different localities, determining the extent of match and mismatch of traits (Toju *et al.*, 2006). This 'geographic mosaic theory of coevolution' helps in understanding the process of coevolution occurring at various spatial levels, from local populations to species ranges. The evolution of most species has involved interacting with other species. Almost all studies of coevolution among species have shown geographic differences in traits

acquired via natural selection from interactions with each other (Thompson, 2005). Studies of *Drosophila melanogaster* and its parasitoids illustrate this mosaic structure in the form of defence and counter-defence across the landscape. *Drosophila* populations vary geographically in the way they defend themselves against two parasitoid species, and both parasitoids vary geographically in the way they mount their counter-defence (Kraaijeveld *et al.*, 1999). Plant species generally vary among populations in their defences against herbivores or the way they reward mutualists (Linhart *et al.*, 1996). Herbivore populations also diverge in their counter-defences to specific plants, as well as in their level of specialization to single or multiple plant taxa (Thompson, 2005).

Phenotypic selection might be asymmetric in different patches, even over short distances of a few metres (Stewart *et al.*, 1987). Toju *et al.* (2006) showed phenotypic variation among populations in the attacks of camellia weevil and defence of Japanese camellia. Studying the floral morphology of *Alkanna orientalis* in Sinai showed spatial differences in the direction of selection among and within wadis (Gilbert *et al.*, 1996). Geographical differences in selection caused by interspecific interactions, along with its output in local adaptation, constitute a genetic and ecological template to understand the way in which coevolution happens - the geographic mosaic theory of coevolution (Thompson, 2005).

Studying comparative phylogeography enhances understanding of how the distribution of coevolving species changes over time. A comparative study of their genetic patterns might give a hint of differences in distribution or population size caused by extrinsic factors affecting the whole community, or alternatively suggest that each species has been affected independently by different processes. Diverse comparative studies across many groups of species have supported the idea that interacting species

share a common phylogeographic pattern (Hewitt, 1996, 1999; Sc *et al.*, 2006; Smith *et al.*, 2011), while others suggest that each species has a unique independent biogeographic history (Sunnucks *et al.*, 2006; Solomon *et al.*, 2008; Moussalli *et al.*, 2009). Why are such differences found among these studies of organisms sharing similar ecological habitat? Smith *et al.* (2011) put forward several possibilities. First, the physical landscape may influence the phylogeographic patterns among taxa, e.g. greater possibilities of corridors and/or refugia mean it becomes less likely that the interacting species will share common phylogeographic patterns. Second, the extent to which coevolving organisms are likely to share similar phylogeographic patterns may be influenced by the extent to which they share relevant characteristics, such as dispersal ability and special habitat requirements. Third, the strength of the ecological relationship between the organisms, e.g. generalists *vs.* high specialists: interacting specialists should be more likely to have a shared evolutionary history because their mutual interdependence.

Highly specialized or obligate pollination mutualisms constitute an excellent system to test the hypothesis of whether coevolving organisms respond to extrinsic environmental factors in a similar manner. This is can be achieved by studying the phylogeographic and demographic patterns of both species using molecular data. Smith *et al.* (2011) studied changes in the range size of the Joshua tree (*Yucca brevifolia*) and four associated specialist yucca moths (Prodoxidae) using molecular data, species distribution modelling methods, and the simulation of molecular data under different demographic histories. Their results suggest that this group of mutualists may have a common population history, reacting simultaneously to environmental fluctuations. In contrast, Espíndola *et al.* (2014) studied the phylogeography of *Trollius europaeus* and

three of its specialized fly pollinators. They concluded that each fly species reacted independently to the forces experienced in common, causing varying levels of compatibility with the genetic pattern of the plant.

1.4 Thesis outline

The ultimate goal of this study is to understand the genetic structure of the coevolved system of a desert plant (*Alkanna orientalis*: Boraginaceae) and its main pollinator, the bee *Anthophora pauperata* (Anthophoridae) in order to reach a coherent conservation strategy. *Alkanna orientalis* has many uses in medical preparations, cosmetics, dyes and food colorants (Hammouda *et al.*, 1992; Akçin *et al.*, 2004; Assimopoulou *et al.*, 2006; Tawfik *et al.*, 2007; Esfahani *et al.*, 2012; Bame *et al.*, 2013). It probably reproduces exclusively via seeds and is restricted to outcrossing (Gilbert *et al.*, 1996). It is distributed extensively on the mountaintops of the eastern Mediterranean and Middle East regions, with an exceptionally isolated colony in Algeria; however, it is under human threats in populated areas where the plant can be neglected and destroyed. The plant is the main food source of the solitary bee *Anthophora pauperata* during the main flowering period in the early part of the spring season in Sinai (Egypt), the only known population before this study. I use molecular, ecological and behavioural methods to look at how plant genes are moved by the bee, and the consequences of this for adaptation and diversification. There are limited available molecular and ecological data on *Alkanna orientalis* and its solitary bee pollinator, apart from what has been studied in Sinai. The present study has four main objectives presented in four chapters: -

- Chapter 2 assesses a spectrum of pollinator species to test whether the bee *Anthophora pauperata* is the species most likely to have coevolved/be coevolving with *Alkanna orientalis*, by measuring the visitation rate and pollination efficiency.

- Chapter 3 describes the territorial behaviour of male *Anthophora pauperata* in Saudi Arabia in detail, and assesses the ability of both sexes to move long distances in the difficult landscape, thereby shedding light on the possibilities of gene flow through pollen transfer between habitats.

- Chapter 4 investigates the genetic variation within and am populations of *Alkanna orientalis* to understand its phylogeography on both small and large geographical scales, using maternally inherited *vs.* biparentally inherited genes to reflect gene flow through pollen *vs.* seeds.

- Chapter 5 investigates the genetic variation within and among populations of *Anthophora pauperata*, also with a view to understanding its phylogeography on both small and large geographical scales, again using maternally inherited *vs.* biparentally inherited genes.

Finally, chapter 6 discusses all of the previous ecological and molecular investigations in order to obtain more meaningful explanations of phylogeographical patterns. The ultimate intention of this study is to answer the question of how I can develop conservation strategies for this plant-pollinator system, and to prioritize particular populations. I therefore conclude with a discussion of the conservation implications the work.

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Chapter 2: Flower visitors to *Alkanna orientalis* (Boraginaceae) in Saudi Arabia and their pollination effectiveness

2.1 Abstract

Flower traits filter flower visitors and dictate their efficiency as pollinators. The floral characters of *Alkanna orientalis*, a plant of isolated mountain-top islands in the Middle East, suggest pollination by long-tongued bees, and just such a bee, *Anthophora pauperata*, is known to be its main pollinator in the mountains of South Sinai. To investigate its pollination in the northern Madiyan mountains of north-western Saudi Arabia, I considered the spectrum of visitors to its flowers and their abilities in pollination. In Jabal El-Loz, *A. pauperata* was the most common visitor for both pollen and nectar, and was also an efficient pollinator, transferring pollen to the stigmas. A species of *Megachile* (*Chalicodoma*) is also a common visitor, but only for nectar, as in many other visitors. In terms of pollen deposition, the most effective visitor was *A. pauperata*.

Key words: *Alkanna orientalis*, *Anthophora pauperata*, pollination, coevolution

2.2 Introduction

Pollination processes are regularly considered as a symbiotic relationship where plants pay a reward to flower visitors that deposit conspecific pollen, but they can also be thought of as mutual exploitation rather than symbiosis (Faegri *et al.*, 1979; Waser *et al.*, 1996; Willmer, 2011). Many ecologists and evolutionary biologists consider that these interactions are crucial to understanding plant diversification (Grant *et al.*, 1965; Stebbins, 1970), starting with Darwin (1862), who assumed that the precise floral morphology of orchids is the consequence of coevolution with particular insect pollinators. By the mid-20th century, pollinator specialization was an underlying theme of adaptive radiation among plants at different taxonomic levels (Grant *et al.*, 1965; Dodson, 1980; Crepet, 1983; Galen *et al.*, 1987). For example, Galen (1989) measured pollinator-mediated selection on the flowers of *Polemonium viscosum* in Colorado (USA). Pollinators of *P. viscosum* vary with altitude, but at higher elevation become very specialized on bumblebees. The experiment showed that bumblebees exerted significant selection on the floral phenotype of *P. viscosum* sufficient to explain changes in flower morphology over the altitude gradient.

Many later studies support Darwin's model of pollinator-mediated selection connected to alterations of flower morphology and floral display (Alexandersson *et al.*, 2002; Sandring *et al.*, 2009; Sletvold *et al.*, 2010). Floral characters evolve to provide suitable characteristics that meet the needs of their pollinators and thus maintain plant fitness (Faegri *et al.*, 1979; Mayfield *et al.*, 2001). Selection on floral traits is correlated with the pollinator types to be attracted, and once specialization of the flower is attained, it will be pollinated by a particular set of visitors (Grant *et al.*, 1965; Stebbins, 1970; Faegri *et al.*, 1979).

The selective influence of flower visitors upon floral traits depends on their pollination effectiveness, the extent to which the visits of a pollinator result in plant reproductive success, either by the male function of spreading pollen to other individual plants, or via the female function of setting seed (Herrera, 1988; Thompson, 2005). I concentrate here on the female function: there are many factors that can affect the pollination process and lead to failure of seed production. Higher risk is associated with pollination limitation, i.e. where reproductive success depends on pollen quantities delivered to flowers, that might therefore be delivered in inadequate amounts, at inappropriate times or of poor quality (Wilcock *et al.*, 2002; Harder *et al.*, 2010). The total deposition of pollen received by a plant may adversely affect its fitness through influencing the number, size and quality of seeds produced (Zimmerman *et al.*, 1988). Pollination effectiveness therefore means the combination of efficacy, the total deposition of pollen during a single visit and its relationship to plant fitness (Herrera, 1989; Johnson, 2006), which can be measured, together with the frequency of flower visits by that pollinator (Stone, 1996; Gómez *et al.*, 1999). The most straightforward way to calculate an index of pollination efficacy is to count the number of pollen grains on the stigma of a flower after a single visit, and to count the number of mature seeds set in the resulting ovary (Stebbins, 1970; Schemske *et al.*, 1984; Inouye *et al.*, 1994; Dupont *et al.*, 2004; King *et al*., 2013). Thus, pollinator efficacy along with visitation rate defines pollination effectiveness through seed production, and consequently selection on floral traits from the female function occurs via this mechanism (Aigner, 2001).

The aim of this study is to consider the ability of visitors to deposit pollen on the stigma of *A. orientalis* flowers in northwestern Saudi Arabia. I test whether the plant needs insects for pollination, and measure the frequencies of visits by the different

visitors and the amount of pollen deposited by single visits; I also measure longer term seed production. The hypothesis is that, as in the nearby mountains of South Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Stone *et al.*, 1999), long-tongued bees are the main or only efficacious pollinator compared with other possible flower visitors because of their physical match with flower morphology.

2.3 Materials & Methods

The study took place throughout March and April 2015 at Jabal El-Loz (Madiyan mountain range, Tabuk region) in the northern part of the western highlands of Saudi Arabia (N 28° 43.452' E 35° 19.417'). The mountainous topography is similar to that of St Katherine in South Sinai (Egypt), 140 km away across the Gulf of Aqaba, where the *Alkanna-Anthophora* system has been studied previously (Fig. 2.1) (Willmer *et al*., 1994; Gilbert *et al*., 1996; Stone *et al*., 1999; Nuttma *et al*., 2006). It is a barren area with a maximum altitude of 2580 m. The climate is normally exces vely dry, with hot rainless summers and cool winters. The mean annual rainfall is very low, with a mean of 20-40 mm in the northwest (Hasanean *et al.*, 2015) but regularly with long periods of drought throughout the year broken by high-intensity rain and floods. The mean annual temperature is about 22.5 °C (Almazroui *et al.*, 2013). The landscape is marked by rugged mountains with intervening steep-sided valleys (wadis). Seven distinct but geographically close wadi systems, each with 1-3 sites separated by more than 500 m, were deliberately chosen to be able to detect small- and large-scale movements (Fig. 2.2): they were labelled by wadi number and plot letters within wadis.

The study focussed on *Alkanna orientalis* (L.) Boiss. (Boraginaceae), a medicinally significant perennial herb with yellow flowers, glandular-sticky leaves, a shrubby base and a coloured root that grows in dry places (Fig. 2.3a-c) (Tutin *et al.*, 1972). It is distributed extensively on the mountaintops of the eastern Mediterranean and Middle East regions, with an exceptionally isolated colony in Algeria (Fig. 2.4) (Gilbert *et al.*, 1999; Nuttma *et al.*, 2006). The genus *Alkanna* Tausch has about seventeen species (Mabberley, 2008), all distributed from the southern Mediterranean eastwards to Iran. The sticky and irritating glandular hairs are a defence against the leaves being eaten by herbivores, which is an effective strategy unless the leaves are very dry (Gilbert *et al.*, 1996; Gilbert *et al.*, 1999). In Sinai, *A. orientalis* grows at elevations higher than 1500 m above sea level (Gilbert *et al.*, 1996), and similarly in northwestern Saudi Arabia. In southwestern Saudi Arabia (the Asir mountain range), the plant occurs only at even higher elevations, starting from about 2000 m above sea level. It grows up to about 1 m in diameter and about 25 cm in height, with up to several hundred yellow flowers containing nectar and pollen, the main nutrient source for *A. pauperata* (Fig. 2.3D) (Stone *et al.*, 1999). The fairly large trumpet-shaped flower is protandrous, with three upper and two lower bilocular anthers, and a single gynobasic style; the flowers grow on a determinate scorpioid cincinnus. The ovary of *A. orientalis* contains four ovules. Following successful pollination, each ovule develops one seed, and hence each flower can yield a maximum of four seeds, easily counted after their formation (Fig. 2.3E). Gilbert *et al*. (1996) suggested that the plant reproduces exclusively via seeds and is an obligate outcrossing species.

To identify flower visitors and to define their visitation frequencies, observations were carried out throughout March 2015 at Jabal El-Loz. Twelve plots of 100 x 100 m

(Fig. 2.2) were chosen in various wadis in areas of high plant density, separated by at least 500 m. All plants were recorded by drawing a map for each plot (see Supplementary Fig S1a-e). Unfortunately, I was only able to finish the study of flower visitors in five plots because of safety reasons as the security situation became unstable in the region. Thus for a 5-day period, each day in a different wadi and plot, I monitored about 25 plants in each plot, starting early in the morning before the sun struck the plants. All times quoted here are in local time (GMT+2). Every 15 mins until the went down (or insect activity stopped), I chose one plant to watch using a random-number generator (an android phone application). On each plant, I counted the number of flowers at the female stage, where the petals were fully open and bright yellow, and observed the plant for 10 mins, counting the number of each type of flower v visiting the plant, the number of flowers each visitor visited, and whether visits were legitimate or illegitimate and for pollen, nectar or both. In addition, I noted down any hostile interactions among the flower visitors. A hostile interaction arose when an animal behaved aggressively to evict others from flowers or to stop them approaching open inflorescences. On this plant, such aggressive behaviour is mainly prevalent in territorial anthophorids (Willmer *et al.*, 1994). The microclimate (relative humidity, temperature and wind speed) was assessed during the time of observations, recorded using a Skywatch Atmos Thermo-Hygro-Anemometer.

I used R for statistical computing (version 3.2.0: R Core Team, 2013) for va tests among visitation rate. The square root of the number of visited flowers was calculated to achieve normality.

Pollinator efficiency was assessed by counting the number of pollen grains deposited on an unvisited stigma from a single visit (Inouye *et al.*, 1994); I was unable to

distinguish self- from cross-pollen in these experiments. From the twelve plots, I chose three (1A, 2A and 3A) with high densities c *A. orientalis*. On each of 30 random plants per plot in three plots in each of three different wadis, I bagged three buds on three different flowering stems using rough tulle netting, which allows air to circulate but prevents visitors; bags remained in place until flowering had finished. In placing the netting, I tried to avoid it touching (and thus potentially pollinating) the flowers. Each of the three flowers on each plant was left until the fifth day or the flower had fully opened, and then each was randomly allocated to one of the following treatments: (1) always closed, then picked and pollen grains counted on the stigma; (2) bag left open to inse visits, then picked and the pollen counted; (3) bag removed, inflorescence picked and placed in water in a tube and offered to visiting insects until visited once by a known visitor [normally *Anthophora*], and then the pollen counted. Pollen grains were measured on the receptive part of the stigma using a field microscope at . I tested for differences in the average amounts of pollen deposited among the three treatments using a one-way ANOVA followed by a Tukey post-hoc test; similar tests were carried out to distinguish differences among visitor taxa in the amounts of pollen deposited. To achieve normality, the data (number of pollen grains deposited) were log-transformed (log +1).

Having picked the experimental flower offered to known visitors, it was of course not possible to count the seeds set as a consequence of the single visit. Instead, the time-course of seed production through the season up to the time of the study was evaluated by counting the number of seeds on every flower remnant down the flowering stem, starting from the top one actually in full flower (numbered 0). I can assume a uniform rate of flower production (which is of course rather unlikely), then

simultaneously flowering flowers can be aligned back through time. Seeds were recognized by their large size, counted and categorized by colour (white [immature], green [mature], or brown [old dry seeds]); they could not be counted in the first few flower remnants because sufficient time had not elapsed for the plant to fill them.

2.4 Results

Flower visitors and visitation frequency were tested in five plots in four wadis with different elevation (Table 2.1). Among plots, the microclimate was slightly different, but there was no obvious difference concerning visitor activity. At each plot (Fig -e), ambient temperatures increased rapidly after sunrise or at the time when the sun reached the plot and dropped rapidly after each plot became shaded. Ambient temperatures escalated from a minimum of 2-3 °C to reach a maximum of 27-30 °C. Relative humidity reduced from the highest levels of 4 -65 % early in the morning unti it reached lows of 5-15 % by midday, rising again by late afternoon. Wind speed was mostly zero early in the morning and accelerated during the day until it reached -25 km/h. Each plot has its own unique topography, which determined the amount of direct sunlight received by a particular plant over a specific period of time. The plot least shaded by the mountains was wadi 2 site B (plot 2B) which received direct sunlight for the longest period each day (from ≈ 06:45 – 18:25 hours daily). In contrast, plot 3A was the most shaded and received direct sunlight for the shortest period (from ≈ 08:55 – 18:15 hours daily). Such topographical differences dictated that *Alkanna* patches received sunlight in plot 2A earlier than 4A or 1A (07:15, 07:50 and 08:15 respectivel and were shaded earlier in 4A than 2A or 1A, the last plot to lose the sunlight (17:40, 18:00 and 18:40 respectively

Overall, twenty-four species of visitors were seen and/or sampled on flowers of *A. orientalis* out of twenty-six arthropod species seen on the plant (Table 2.2): some photographs are shown in Figure S2. These species made regular visits to the flowers but show significant differences in activity and visit frequency (Some anthophorid species were unfamilier and are currently awaiting assessment and description by Chris O'Toole, which may show that they are new to science). From 54 h of observation samples, 576 individuals of ten species were seen on 129 plants (with about 10930 flowers) across the different plots/wadis (Table 2.3). These ten species out of the total of twenty-six were recorded as regular visitors during the experiment time of 1(-minute observation periods. The most frequent visitor was *A. pauperata*, which represented 75% of the total flower visits seen. The second most frequent visitor was *Megachile* (*Chalicodoma*) sp. 1 which made 13% of the total flower visits. *Eucera* (*Synhalonia*) sp. 1, *Anthophora cyrenaica*, and *Megachile* (*Chalicodoma*) *parietina* were relatively minor but regular visitors. A number of other species were recorded making a few visits to the flowers (Table 2.3). The pattern of visitation started early in the morning with a few individuals of one or two species (usually *A. pauperata* and *Xylocopa*), increasing to the highest density of visits at midday, and then decreasing gradually in the afternoon until again there were just a few individuals of one or two species (Fig. 2.6).

There was a positive correlation between the density of flowers on a plant and the overall number of visits to its flowers across all sites (Fig. 2.7: $r = 0.482$, $n = 129$, $P << 0.001$), and there was also a positive correlation with the rates of visits per flower ($r = 0.215$, $n = 129$, $P << 0.014$). In an ANCOVA involving three predictors of the number of visits (Table 2.4), flower density dominates (explaining 28% of the variation), with pl

differences also important (16%): although highly significant, time of day effects are relatively minor (13%

Male *A. pauperata* showed unimodal activity, with most of their foraging early in the morning (Fig. 2.8), and only for nectar. Female *A. pauperata* clearly showed bimodal activity in all plots, with obviously separate morning and afternoon peaks (Fig. 2.8). This bimodal patter was much stronger for pollen collectors because the peaks were separated by nearly zero activity. Female *A. pauperata* were seen digging nests near *Alkanna* and other plants (recorded on video) in the middle of the day, again emphasizing the bimodal foraging activity pattern (Fig. 2.9). Female *A. pauperata* apparently spent most of the middle of the day (about 3 h between 10/11 and 13/14 h [GMT+2]) underground in their nests.

In the pollination efficiency experiment, significant differences in the amounts of pollen deposited were found among the pollination treatments (Table 2.5). As expected, unvisited flowers received less pollen than those that had had a single visit, which in turn received less than flowers open to many visits (Fig. 2.10A). There were also significant differences among wadis (Fig. 2.10B). For the number of pollen grains deposited after a single visit, there were some significant differences among visitor species (Kruskal-Wallis test $df = 6$, $P = 0.03746$) (Fig. 2.11A & 2.11B), despite the fact that sample sizes were small for some of the less common visitors.

The frequency of visits to flowers by visitor taxa, and the mean number of visitors are shown in Figure 2.12. These illustrate significant differences among taxa, and that visits from male *Anthophora pauperata* are less frequent than visits by females.

Seed production was assessed at the three plots with the highest density of *A. orientalis*. There was a substantial variety in the patterns (Figs. 2.13 & 2.14), perhaps

reflecting the beginning and possibly the end of seasons at the various plots. At plot 1A, the oldest flowers were unpollinated and the proportion of pollinated flowers increased with time. At plot 2A, the most recent flowers remained unpollinated and the probability of pollination increased with age except in the oldest flowers, where the probability fell back to zero. At plot 3A, the most recent flowers remained unpollinated, while through time the probability of pollination increased and then decreased again. Overall across wadis (Fig. 2.14), the proportion of pollinated flowers increased with age and gradually decreased again; the most recent two flowers (numbered 0 and 1) were recorded as unpollinated probably because the seeds had not had enough time to develop. Over the two weeks of the field study, it was obvious that the overall frequency of visits by all species including *Anthophora* (which made most of the visits) was decreasing (Fig. 2.15).

Table 2.1: Studied plots with elevations and location at Jabal El-Loz (Madiyan Mountains range, Tabuk region, NW Saudi Arabia).

100 x 100 m plot		**Location**	
plots	elevation/m	Latitude	Longitude
1-A	2042	N 28° 43.452’	E 035° 19.417’
2-A	1976	N 28° 44.274’	E 035° 19.370’
2-B	2006	N 28° 44.264’	E 035° 19.282’
3-A	1952	N 28° 44.420’	E 035° 20.172’
4-A	1655	N 28° 43.712’	E 035° 21.526’

Table 2.2: Visitor taxa seen on the flowers and plants of *Alkanna orientalis* at Jabal El-Loz in March-April 2015. Photographs of some of these taxa are shown in Figure S2.

No.	order	family	species
1	Araneae	Oxyopidae	*Peucetia arabica* Simon (on leaves and stem only)
2	Coleoptera	Scarabaeidae	*Oxythyrea* sp. 1 (illegitimate visito
3	Diptera	Bombyliidae	Bombyliid fly
4	Diptera	Nemestrinidae	*Nemestrinus rufipes* Olivier [or possibly *Nemestrinus laetus* Loew]
5	Diptera	Syrphidae	*Eupeodes* (*Metasyrphus*) sp?
6	Hymenoptera	Apidae	*Anthophora cyrenaica* Gribodo
7	Hymenoptera	Apidae	*Anthophora dispar* Lepeletier
8	Hymenoptera	Apidae	*Anthophora disparalis* Friese
9	Hymenoptera	Apidae	*Anthophora flabellata* Priesner
10	Hymenoptera	Apidae	*Anthophora pauperata* Walker
11	Hymenoptera	Apidae	*Anthophora* sp. 1 (Anthophora cf. *hermanni* Priesner) [possibly an undescribed species]
12	Hymenoptera	Apidae	*Anthophora* sp. 2
13	Hymenoptera	Apidae	*Anthophora* sp. 3
14	Hymenoptera	Apidae	*Anthophora* sp. 4
15	Hymenoptera	Apidae	*Anthophora* sp. 5
16	Hymenoptera	Apidae	*Amegilla nubica* Lepeletier
17	Hymenoptera	Apidae	*Eucera* (*Synhalonia*) sp. 1
18	Hymenoptera	Apidae	*Megachile* (*Chalicodoma*) *parietina* (Geoffrey)
19	Hymenoptera	Apidae	*Megachile* (*Chalicodoma*) sp. 1
20	Hymenoptera	Apidae	*Thyreus* sp. 1
21	Hymenoptera	Apidae	*Thyreus* sp. 2
22	Hymenoptera	Apidae	*Thyreus* sp. 3
23	Hymenoptera	Apidae	*Xylocopa (Proxylocopa)* sp. 1
24	Hymenoptera	Vespidae	Solitary vespoid sp. 1 (sitting on leaves only)
25	Lepidoptera	Sphingidae	*Hemaris croatica* Esper
26	Lepidoptera	Papilionidae	*Papilio* sp. [probably *saharae* Oberthür]

Table 2.3: Overall numbers of the visitors to the flowers of *Alkanna orientalis* in five plots in Jabal El-Loz, Saudi Arabia in March-April 2015. The left side of the table shows the dates of observations and the plant density for each 100x100 m plot that was studied. The middle part shows the number of observed plants with the total number of flowers at the female stage, the number of individual visitors and the number of flower visits during the observational periods. The right side shows the species of flower visitors, the number of observed individuals and the total number of visits.

100X100m			observed plants				observed species		
date	**sites**	**plant density**	**no. of 10-min sample**	**flowers at female stage**	**no. of visitors**	**no. of flower visits**	**species**	**frequency**	**no. visits**
19/03/2015	1-A	38	23	2322	187	3100	*Anthophora pauperata* Walker	166	2799
							Megachile (*Chalicodoma*) sp. 1	10	56
							Eucera (*Synhalonia*) sp. 1	8	226
							Anthophora cyrenaica Gribodo	3	19
20/03/2015	2-A	77	24	2645	136	2141	*Anthophora pauperata* Walker	101	1510
							Megachile (*Chalicodoma*) sp. 1	25	535
							Anthophora cyrenaica Gribodo	4	61
							Megachile (*Chalicodoma*) *parietina* (Geoffroy)	6	35
30/03/2015	2-B	20	31	2116	166	2029	*Anthophora pauperata* Walker	119	1462
							Megachile (*Chalicodoma*) sp. 1	34	374
							Eucera (*Synhalonia*) sp. 1	6	86
							Anthophora cyrenaica Gribodo	1	2
							Anthophora flabellata Priesner	1	1
							Bombyliid fly	1	24
							Thyreus sp. 1	4	80

100X100m			observed plants				observed species		
date	**sites**	**plant density**	**no. of 10-min sample**	**flowers at female stage**	**no. of visitors**	**no. of flower visits**	**species**	**frequency**	**no. visits**
01/04/2015	3-A	67	25	2052	46	953	*Anthophora pauperata* Walker	29	604
							Megachile (*Chalicodoma*) sp. 1	3	36
							Eucera (*Synhalonia*) sp. 1	3	47
							Anthophora cyrenaica Gribodo	7	243
							Anthophora flabellata Priesner	4	23
02/04/2015	4-A	35	26	1795	41	521	*Anthophora pauperata* Walker	19	248
							Megachile (*Chalicodoma*) sp. 1	4	33
							Eucera (*Synhalonia*) sp. 1	7	118
							Anthophora cyrenaica Gribodo	6	61
							Bombyliid fly	3	25
							Thyreus sp. 2	1	3
							Thyreus sp. 3	1	33
Total	—	237	129	10930	576	8744	—	576	8744

Table 2.4: ANCOVA table for the effect of three predictors on the number of visits to flowers of *Alkanna orientalis*; one predictor is a covariate (the number of flowers at the female stage), and the others are factors.

Predictor	Df	Sum SQ	Mean SQ	F value	Pr (>F)
Flowers at female stage	1	38.49	38.49	72.2	<< 0.001
Time (hour)	11	17.55	1.60	3.0	0.001
Plot	4	22.77	5.69	10.7	<< 0.001
Residuals	112	59.74	0.53		
Total	128	138.55			

Table 2.5: ANOVA of the number of pollen grains delivered to the stigma of *Alkanna orientalis* as predicted by wadi and pollination treatments. The test was done by sequential deletion, with Poisson errors, tested by Chi-squared.

Effect	*Df*	X^2	*P*
pollination treatment	2	26.3	<< 0.001
wadi	2	65.4	<< 0.001
treatment : wadi	4	7.2	0.1



Figure 2.1: St Katherine in South Sinai (Egypt), where the only other study of *Alkanna* and *Anthophora* took place, and Jabal El-Loz, Tabuk region (north-west Saudi Arabia), where the present work occurred, separated by the Gulf of Aqaba. There is a distance of about 140 km between the study sites.



Figure 2.2: Study plots (red pegs) in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The white lines show the path of the wadis. The yellow pegs show plots that were established but unable to be used because of security considerations.

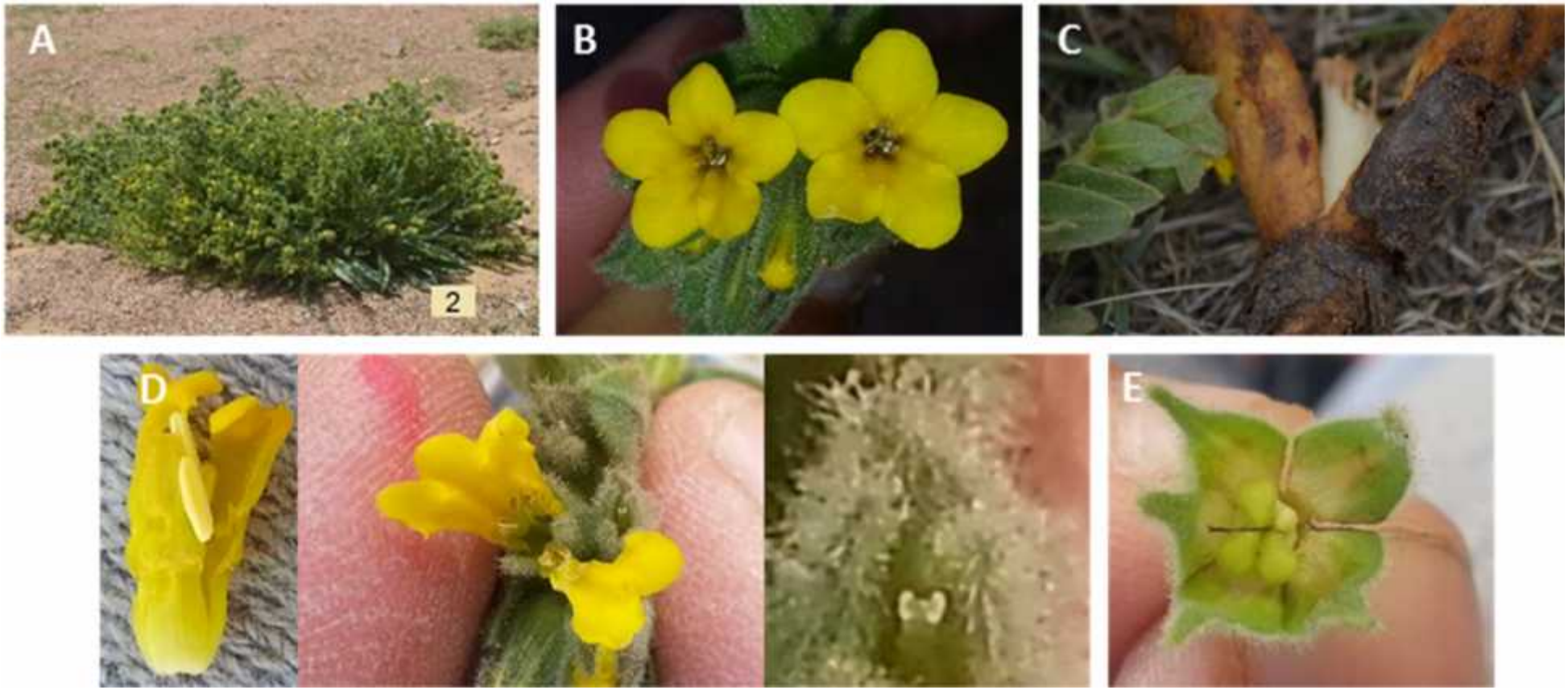


Figure 2.3: Habitus of *Alkanna orientalis*: (a) a typical plant with several hundred yellow flowers; (b) the flowers with their bracts covered with glandular hairs; (c) the red-coloured root; (d) the bilocular anthers, and single gynobasic style; (e) seeds forming after flower abscission.

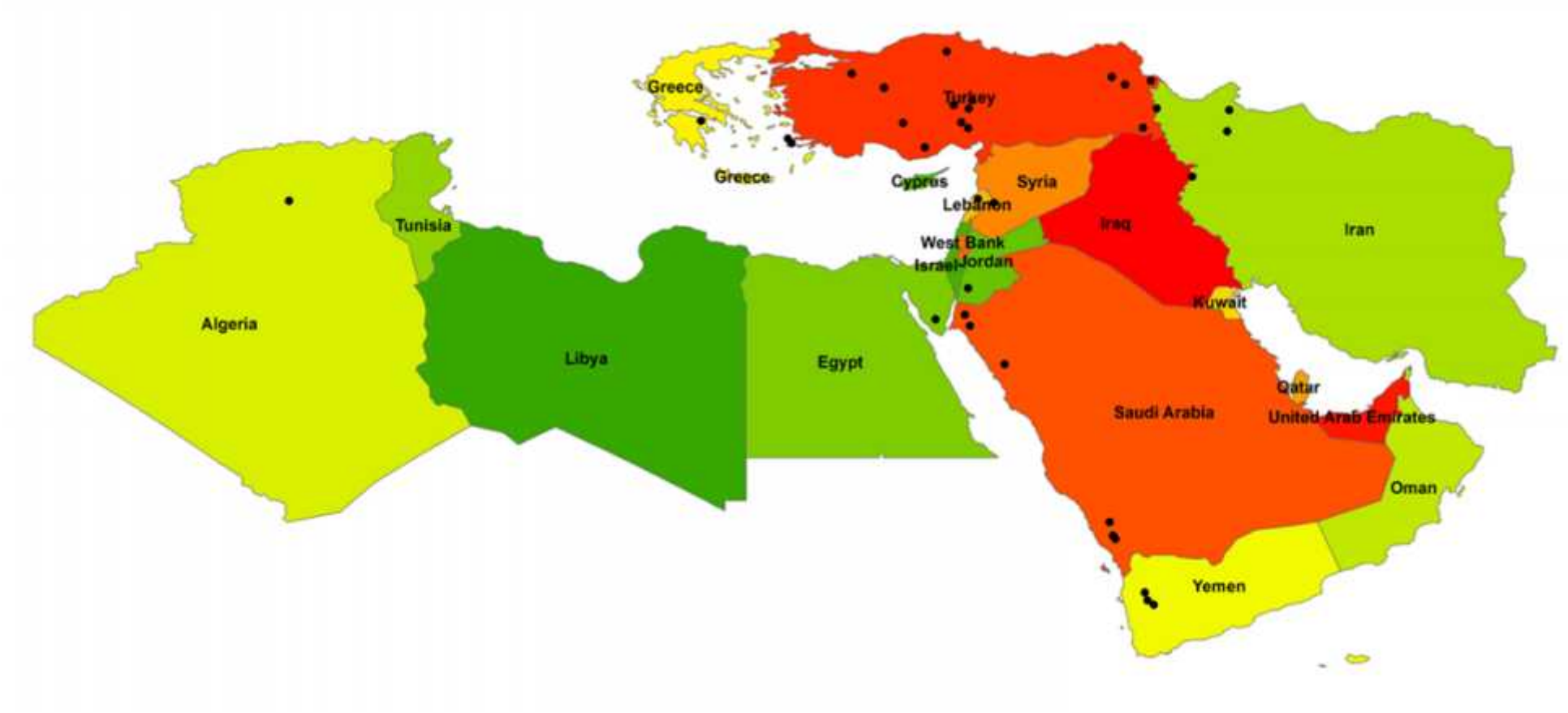


Figure 2.4: World distribution of *Alkanna orientalis*.

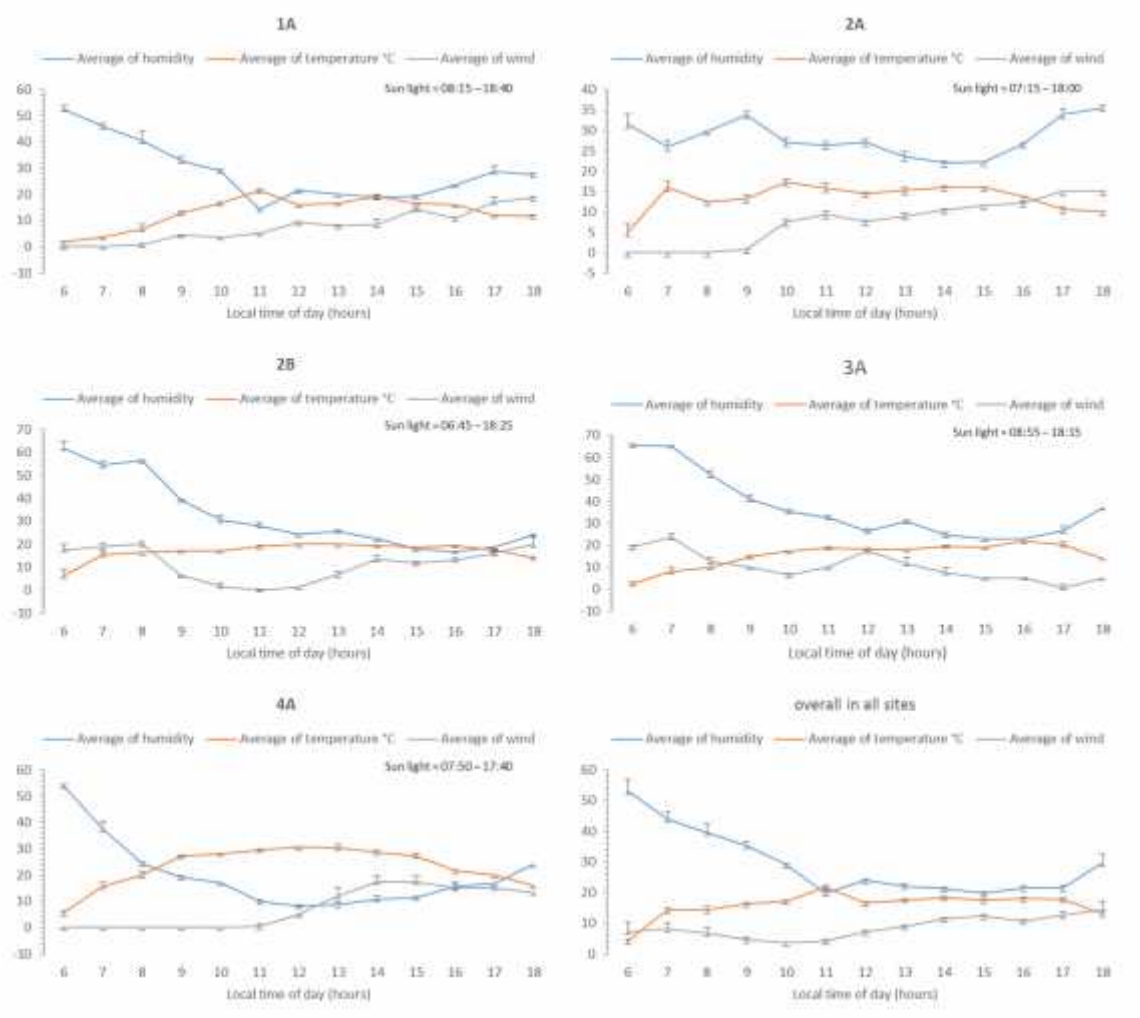


Figure 2.5: Mean (± se) microclimatic conditions (relative humidity [%], temperature (°C), wind speed [km/hr]) for each hour of the day (GMT+2) for each plot, together with the overall mean values across plots at Jabal El-Loz. Each site shows the approximate time of receiving sun light.

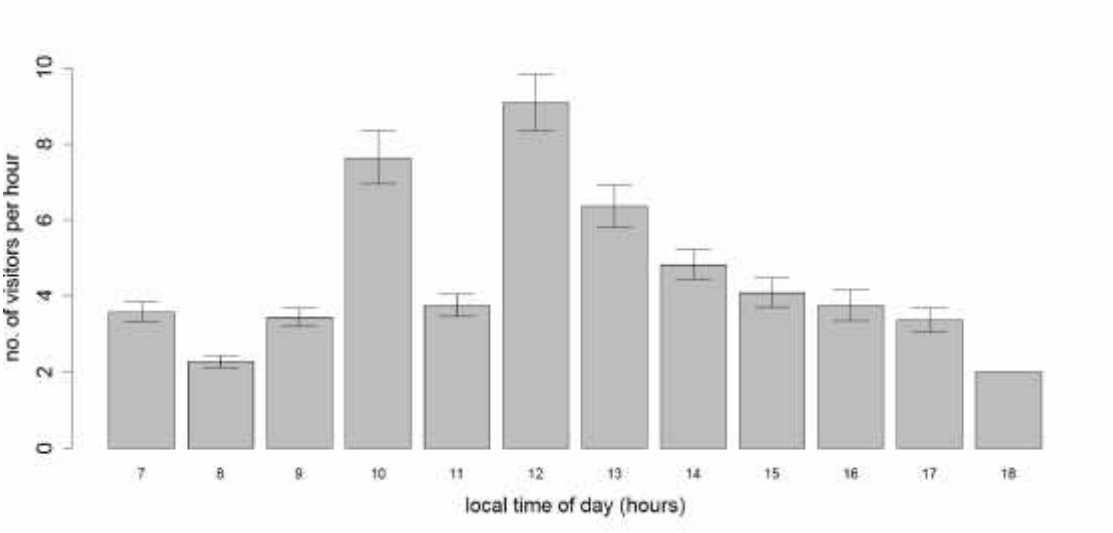


Figure 2.6: The pattern of activity (no. of visitors per 10-minute observation) of all type visitors to flowers of *Alkanna orientalis* by local time of day (GMT+2) across five plc during March-April 2015.

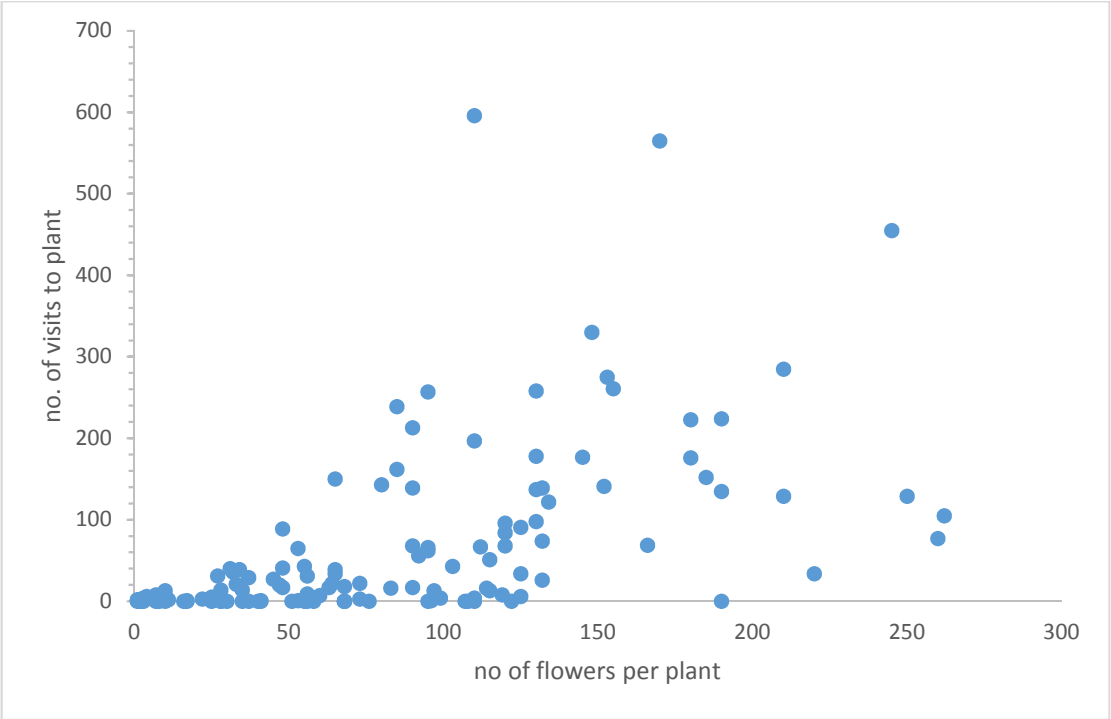


Figure 2.7: The correlation between the number of flowers per plant of *Alkanna orientalis* and the number of visits by insects to their flowers, from timed observations during March-April 2015.

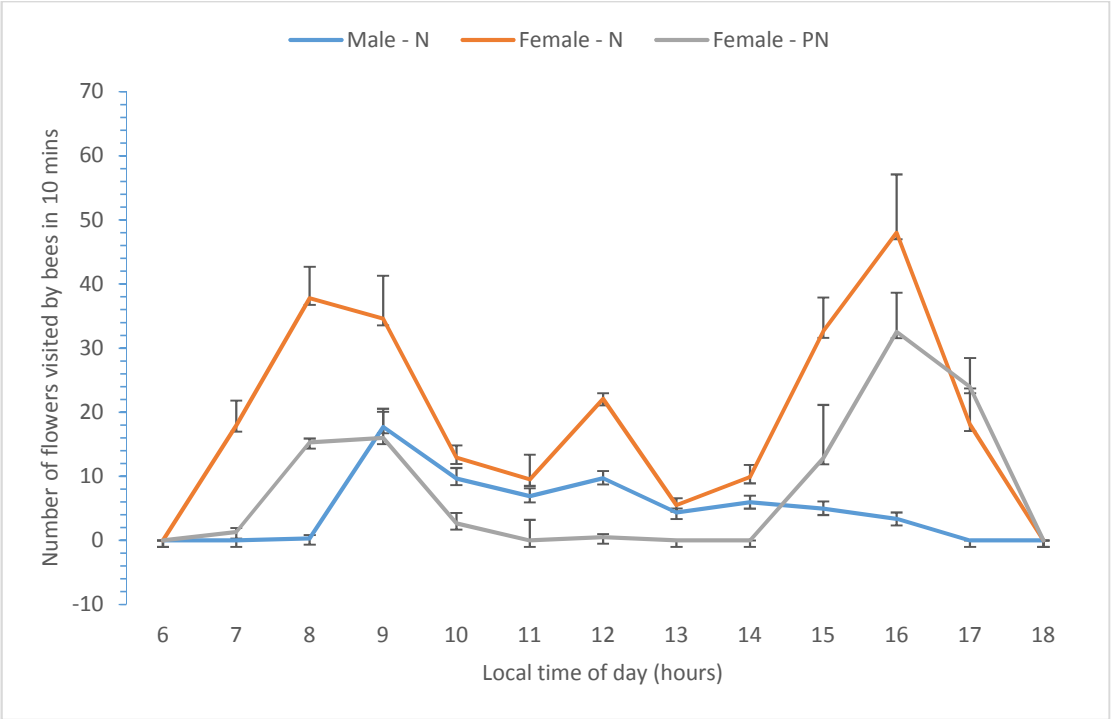


Figure 2.8: Overall pattern (means ± se) of the foraging of male and female *Anthophora pauperata* during the day (GMT+2) across the five studied plots during March-April 2015 (N = nectar only; PN = collecting pollen & nectar).



Figure 2.9: Female *Anthophora pauperata* digging nests during the middle of the day. (left) 13 March 2015 from wadi 1B, under *Artemisia herba-alba*; (right) 02 April 2015 from wadi 4A under *Alkanna orientalis*. Both were observed at Jabal El-Loz, Tabuk region (north-west Saudi Arabia).

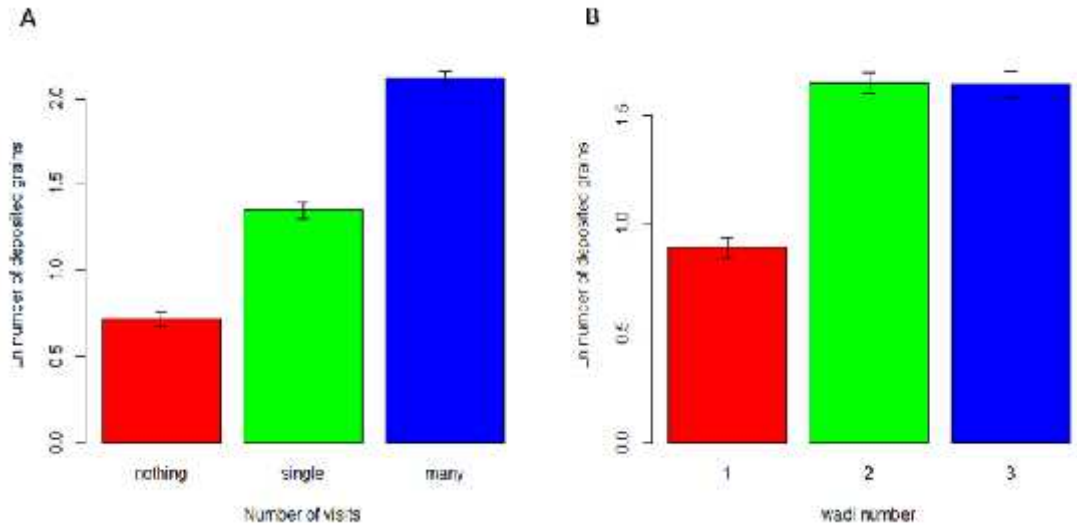


Figure 2.10: Mean (± se) number of pollen grains deposited (A) among the three treatments of no visits, a single visit, and open visitation (many visits); and (B) among the three wadis used in the experiment.

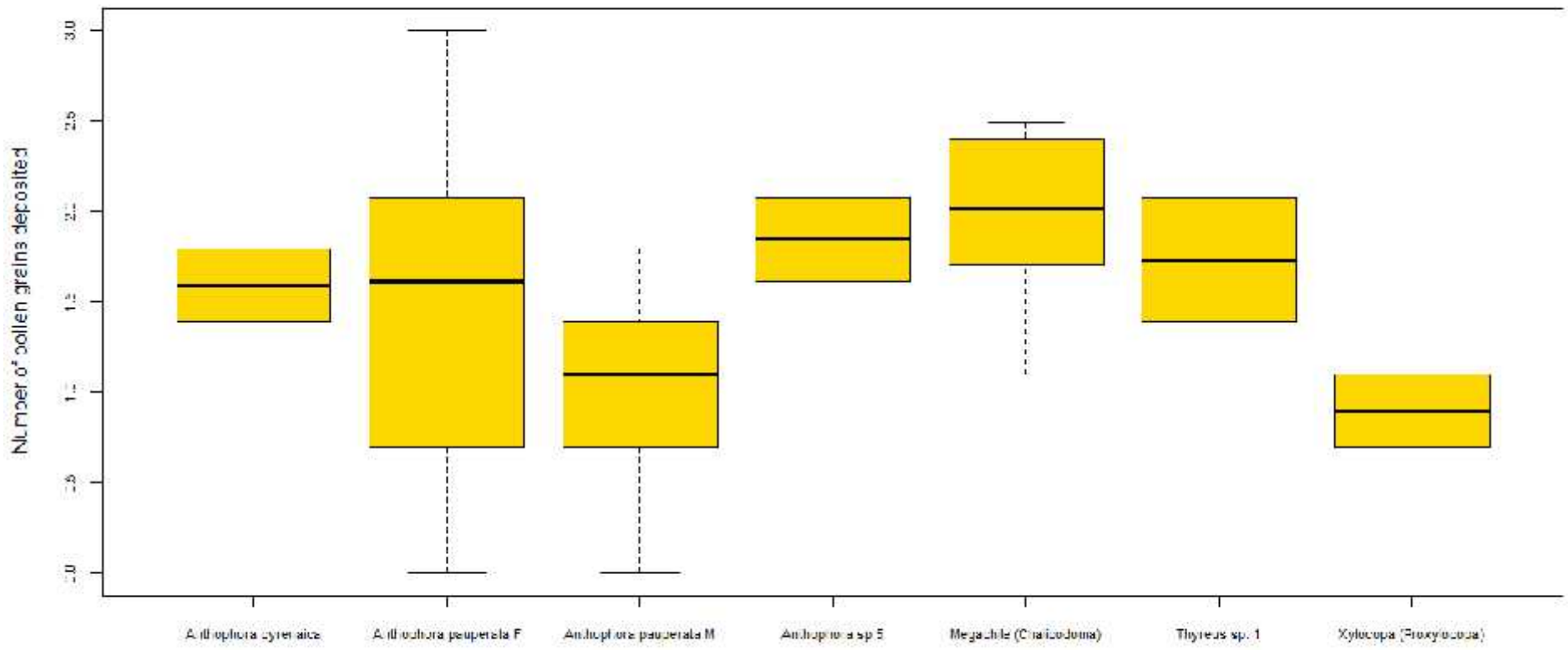


Figure 2.11-A: Number of pollen grains deposited after a single visit by various taxa of visitors (n=90 flowers in total

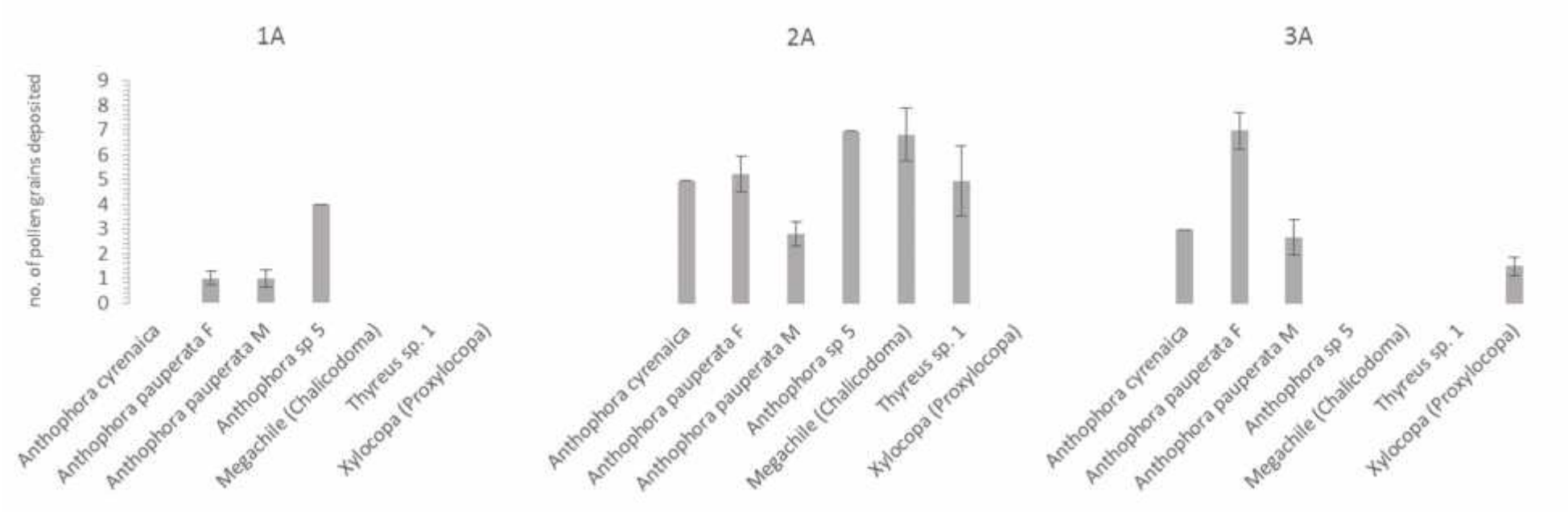


Figure 2.11-B: The number of pollen grains deposited after a single visit split by taxa and site. Some taxa deposited no pollen.

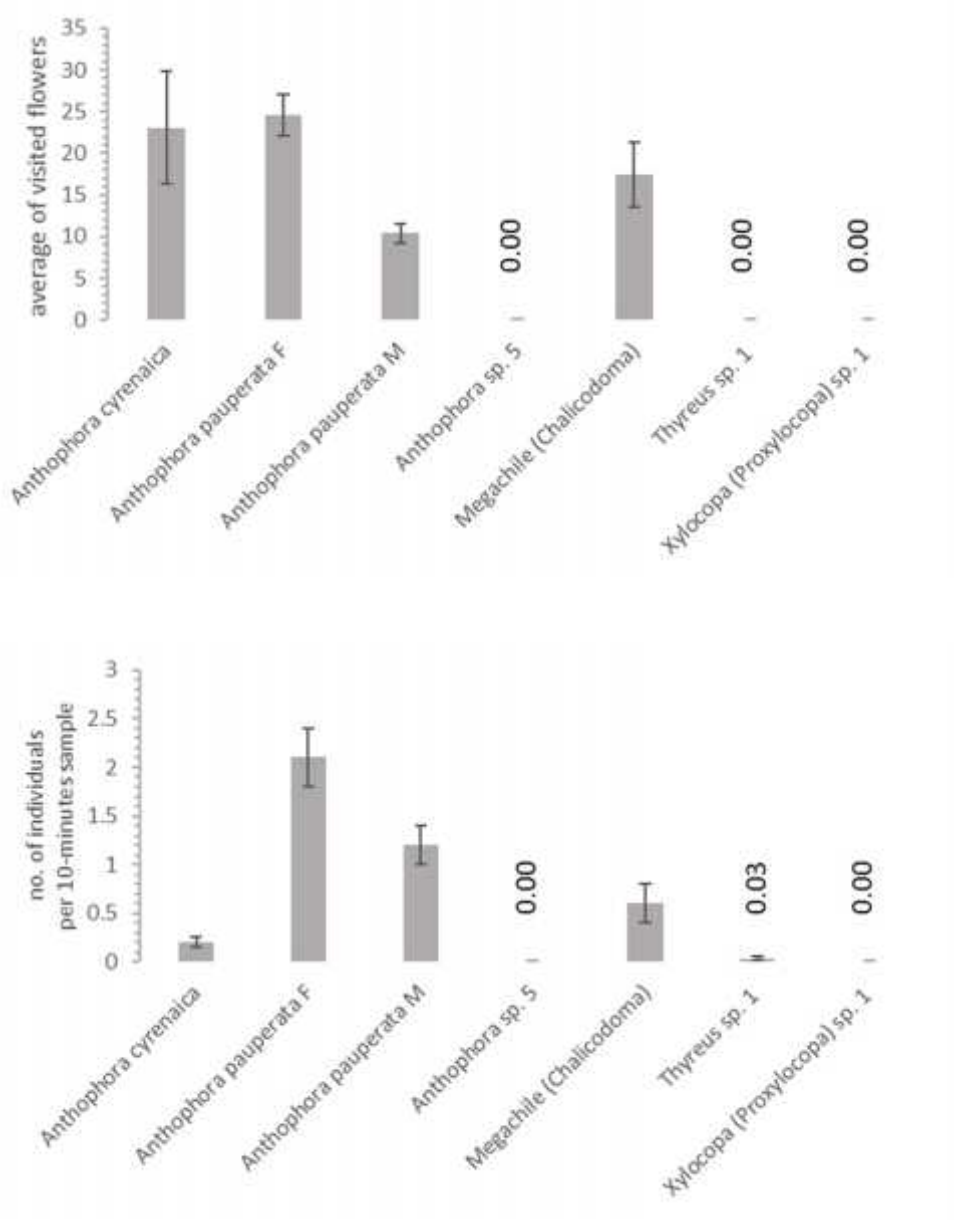


Figure 2.12: (A) Number of flowers visited by various visitor taxa to *Alkanna orientalis* flowers per 10-minute sample (means ± se). The differences among means are significant (Anova with negative binomial errors: χ^2 = 60.9, *df* = 6, p<<0.001), mostly accounted for by the mean for female *Anthophora pauperata* being higher than that of the male (Z = -2.7, *P* < 0.01). The test was done by sequential deletion, with the change in deviance distributed as a chi-squared. (B) Total number of visitors per 10-min sample.

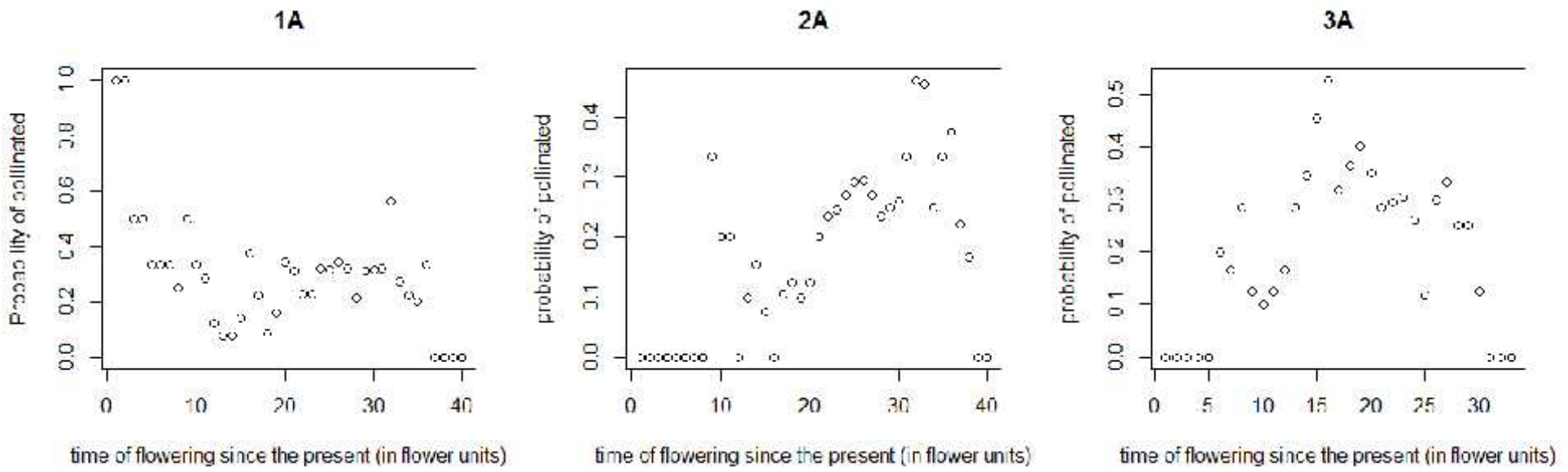


Figure 2.13: Probability of being pollinated (proportion of seeds set) of the flowers of *Alkanna orientalis* against flower sequence, counting from the current flower (0) back down the flowering spike in the temporal sequence of flowering (hence equivalent to time in flower units), for each of the three plots (1A, 2A, 3A).

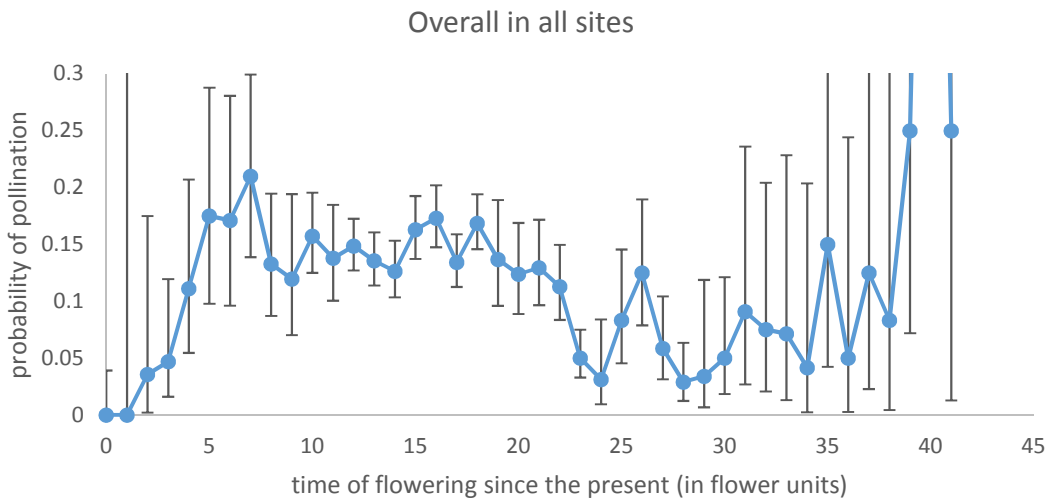


Figure 2.14: The probability of pollination of each *Alkanna orientalis* flower increased in older flowers and then declined in very old flowers (± 95% confidence limits, obtained from the proportions, following Sokal *et al*. (1995).

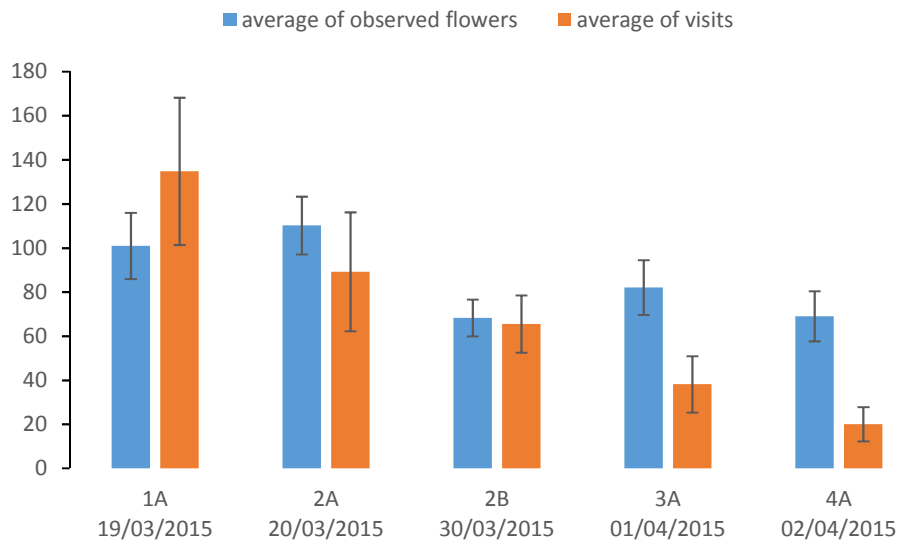


Figure 2.15: This figure shows the number of flowers per plant of *Alkanna orientalis*, and the number of visits per 10-min sample in each of the five sites at Jabal El-Loz (Saudi Arabia), ordered by the date of observation. It is obvious that the number of visits is decreasing through time.

2.5 Discussion

This study confirms that *A. pauperata* is the main visitor and the most efficient pollinator for *A. orientalis*, as in Sinai. The time of flowering of *Alkanna* coincides with the appearance of its pollinator, *A. pauperata*. The sexes of *A. pauperata* behave differently; the females show a bimodal pattern of activity in the early morning and late evening, while the males show a unimodal pattern of activity centred on the middle of the day (from late morning to early evening). As in Sinai, there is limited coincidence in time between males and females. Throughout its active period, *A. pauperata* preferred to visit plants with a high density of flowers.

A number of factors together contribute to the activity patterns of female bees (Stone *et al.*, 1999; Willmer *et al.*, 2004; Kasper *et al.*, 2008), particularly differences in physiology, microclimate fluctuations and the accessibility of food resources. The physiology of foraging at the maximum and minimum ambient temperatures of the local environment may dictate the pattern of activity. The effect might well result in a bimodal activity pattern in environments with a large diurnal temperature range. Bees that possess highly endothermic processes which can reach and maintain the required thoracic temperature for flight at low ambient temperatures (this applied mostly to bees with medium or large body sizes) may not be able to fly in the excessive heat of the midday, eventually abandoning the foraging site (Chappell, 1982, 1984; Willmer, 1983, 1985; Herrera, 1990; El-Moursy *et al.*, 1999; Inouye *et al.*, 2015). Some of these bees may return to the foraging site once the ambient temperature drops, forming a bimodal pattern of activity.

Like other *Anthophora* spp. (Stone, 1993, 1994a; Willmer *et al.*, 1997), *A. pauperata* has the ability to regulate its thoracic temperature in flight independently of the ambient temperature (Stone *et al*., 1999). Similar-sized *Anthophora* species exposed experimentally to similarly low temperatures are able to generate the endothermic heat necessary for flight, but such preflight warm-up will be extremely costly in terms of time and metabolic energy (Stone, 1993). Thus augmenting interr y generated heat with external solar warming will reduce preflight warm-up costs substantially (Stone, 1995 as in *Anthophora plumipes* (Stone, 1994b; Stone, 1995). The activity patterns of female *A. pauperata* in Sinai suggest that temperatures can easily be too low for the bees to generate enough heat by endothermy alone (requiring additional input from basking in sunlight), but also that escaping heat stress is very unlikely as an explanation since maximum temperatures are not very high (max 30 °C) (Stone *et al.*, 1999). As in Sinai, the early morning appearance and late afternoon departure, as well as the overall activity pattern, of *A. pauperata* at Jabal El-Loz differed among studied sites due to the relatively minor differences in the diurnal temperature patterns caused by the site topography, which gives each site its unique diurnal sequence of direct sunlight and shade.

Pevious studies on bees have shown that rain reduces or stops bee activity, but this cannot be the cause of the activity patterns of the bees. During some difficult days of storm and heavy rains at Jabal El-Loz, anecdotally bee activity dropped to zero. This sharp drop in activity seemed to be caused by the physical action of raindrops that halted bee activity (Kasper *et al.*, 2008). Activity increased slightly when the rain stopped, before reducing back to zero again when the rain resumed. However, most days it did not rain, and so this cannot be the cause of the patterns.

There are considerable indications that the reduction in activity of *A. pauperata* during the middle of the day was not because they were evading thermal stress. *A. pauperata* is an efficient thermoregulator (Stone *et al.*, 1999) and is not predicted to be influenced by the midday ambient temperatures recorded in Jabal El-Loz. The bees were able to continue foraging for pollen during the early afternoon when ambient temperatures were quite similar to those faced in the middle of the day (Fig. 2.5). Moreover, although at a low level, females showed the ability to forage for nectar throughout the day (Fig. 2.8). It became apparent that female *A. pauperata* spend the midday period underground tending to their nests. Hence *A. pauperata* may be responding to restrictions on foraging activity, such as the patterns of resource availability; where the resources at that time are scarce (Stone *et al.*, 1999), and therefore it might be better to spend the time in another activity, such as servicing their underground nests. I have seen females digging nests during the midday at different sites (Fig. 2.9).

Local floral resource availability through the day could also be an effective factor generating bimodal activity. Two floral resources are essential for female *A. pauperata*: pollen and nectar. Temporal patterns of resource availability have been well studied (Willmer, 1988; Herrera, 1990; Gilbert *et al.*, 1991; Stone *et al.*, 1996; Stone *et al.*, 1998), including in the flowers of *A. orientalis* in Sinai (Stone *et al.*, 1999). I was not able to examine the patterns of resources availability at Jabal El-Loz in this study, but in Sinai nectar availability did not change significantly through the day (Stone *et al.*, 1999) and this could not therefore be responsible for the bimodal activity pattern of foraging. In contrast, diurnal pollen availability did change significantly, with the peak time being in the early afternoon when the anthers of male-phase flowers dehisced (Stone *et al.*,

1999). Pollen is non-renewable, unlike nectar, which is secreted throughout the life-span of the flower until it gets pollinated (Nuttman *et al.*, 2006). Accordingly, female *A. pauperata* forage for pollen in the early afternoon, gathering what remains in the early morning of the following day (Stone *et al.*, 1999).

All these factors together might modify the timing of nest construction. Female *Anthophora* in appropriate circumstances can construct a nest every day (Batra, 1994; Semida, 2000). *A. plumipes* forages for floral resources during the day and constructs new cells mostly in the evening (Stone, 1994b). *A. pauperata* at Jabal El-Loz displays a pattern that is different from *A. plumipes*, and similar to the pattern described from conspecifics in Sinai (Willmer *et al.*, 1994). Unlike *A. plumipes*, in *A. pauperata* a rapid drop of ambient temperature after sunset not only restricts flight activity, but also restricts movement underground as well. Female *A. pauperata* tend to dig their nests in places surrounded by food resources (Fig. 2.9), carefully choosing the locatic under plants or rocks in dry, coarse sand. The digging of nests (or probably the construction of new cells) occurs in the middle of the day in parallel with the time of insufficient floral resources, when foraging activity would be unproductive because of the relatively low amounts of nectar and absence of pollen (Stone *et al.*, 1999; Semida, 2000).

Bees visited flowers non-randomly, choosing plants with greater number of flowers and flowers with greater resources, by responding to floral characters, ultimately benefitting both plant and visitor (Thomson *et al.*, 1982; Gori, 1983; Kevan, 1983; Weiss, 1991, 1995; Nuttm *et al.*, 2003; Nuttma *et al.*, 2006; Veddeler *et al.*, 2006). Flowers of *A. orientalis* change colour when pollinated: this is correlated with the level of reward availability and is understood by pollinators (Nuttman *et al.*, 2006). They change from bright yellow (containing high levels of reward) to pale yellow/white

(containing significantly fewer rewards), eventually drying and falling off. The floral lifespan to the colour change and floral abscission is determined by the visitation rate of *A. pauperata* (Nuttman *et al.*, 2006). Thus the overall display of plants depends on the proportions of flowers of each colour, which is influenced by being visited. In *A. orientalis*, the floral colour change is associated with pollinating visits: visits or experimental treatments that do not pollinate delay the colour change considerably (Nuttman *et al.*, 2006).

As in Sinai, *A. pauperata* at Jabal El-Loz are influenced by floral colour change and tend to visit the newer bright-yellow flowers. Higher visitation rates were recorded on plants with higher densities of flowers, as many previous studies have concluded (Klinkhamer *et al.*, 1990; Gilbert *et al.*, 1996; Goulson *et al.*, 1998; Ohashi *et al.*, 2002; Makino *et al.*, 2007). Larger floral displays provide higher levels of reward, which means less travel time, and hence more individuals visit. Bumblebees can remember sites where the rewards are high (Cartar, 2004), and can learn to distinguish between flower sizes when this is correlated with the magnitude of the reward (Essenberg *et al.*, 2015). It is possible that *A. pauperata* learns from experience of visiting flowers the connection between flower colour and reward size in *A. orientalis*, thus avoiding the costs of foraging from poorly rewarding flowers and benefitting from revisiting previously highly rewarding ones.

Flowers of *A. orientalis* at Jabal El-Loz were visited by twenty-four species of pollinators with different visitation behaviours (long-tongued bees, butterflies, moths, flies and beetles). *A. pauperata* was the most common visitor and the most efficient pollinator per flower visit. King *et al*. (2013) have clearly stated that “not all visitors are pollinators for a given plant species; a pollinator must deposit sufficient pollen on the

correct and receptive stigma, and that pollen must be conspecific and viable". *A. pauperata* with its long mouthparts was the most adequate visitor capable reaching the nectar situated at the base of the tubular corolla (Stone *et al.*, 1999) and the only visitor capable of collecting its pollen grains. Thus most of the other visitors to *A. orientalis* might be considered as nectar robbers (inauthentic pollinators) foraging for nectar only, and not involved in the pollination process. However, some 'nectar-robber' visitors may also pollinate flowers incidentally. Flowers of *Pitcairnia angustifolia* in the north-central part of Puerto Rico are visited by birds (Bananaquits: *Coereba flaveola*) that behave as nectar robbers (Fumero-Cabán *et al.*, 2007). When the birds are foraging for nectar, the flowers of *P. angusifolia* curve against their bodies and this movement of the flowers almost certainly results in self-pollen being deposited on the stigma.

I found that flowers of *A. orientalis* could be pollinated during a single visit by male *A. pauperata* and some other common visitors who do not normally transfer pollen, such as *Megachile* (*Chalicodoma*) sp. 1, *Anthophora cyrenaica*, *Anthophora* sp. 5, *Thyreus* sp. 1 and *Xylocopa* (*Proxylocopa*) sp. 1 (Fig. 2.12). Despite the fact that some of these visitors were nearly as efficient, or more efficient than female *A. pauperata* in terms of the number of pollen grains deposited per visit (Fig. 2.12), female *A. pauperata* had by far the highest visitation rate (Fig. 2.13) and was clearly the dominant pollinator. Territorial male *A. pauperata* may have had a negative effect on pollination because they were working hard throughout the day to chase away species other than females from approaching the flowers of *A. orientalis*, and in addition they chased away many female *A. pauperata* as well, for reasons that remain obscure. However, other visitors might assume a greater pollination importance at times when the primary pollinators are lacking or at low densities (Navarro, 2000, 2001) and/or over the long-term (Fumero-Cabán *et al.*, 2007).

Plant fitness is the result of successful seed production either maternally on the plant, or paternally by pollinating other plants via pollen transfer. In plant species such as *A. orientalis*, which are obligate outcrossers, the visitation rate plays a significant role in enhancing plant fitness. I have not yet been able to study the role of multiple visits in Jabal El-Loz, but Gilbert *et al*. (1996) have shown that flowers of *A. orientalis* in Sinai do not set as many seeds from a single visit as they set naturally (presumably the result of multiple visits). Thus more visits enhance seed production, implying that the maternal function of the plant is pollen-limited. As a proxy measure I looked at seed production over the longer term by assessing seeds along flowering stems. The variety of patterns I found among the three sites might be due to peculiarities of each location (Table 2.1), or differences in plant density (Table 2.3), or other factors. Nevertheless it is striking that the probability of pollination never reaches very high values, indicating that these plants too are pollen-limited. The diminishing overall probability of pollination in all sites with time (Fig. 2.14) and the decreasing bee density with time (including *Anthophora* which made most of the visits) (Fig. 2.15) are also good indicators that our field season was perhaps a little late for both bees and flowers, and was starting to finish.

This study does not strongly support the hypothesis that *A. pauperata* is the *only* pollinator of *Alkanna* able to deposit pollen on the stigma. Some other species have the ability to do so, nearly as efficiently or even more efficiently than *A. pauperata*. However, *A. pauperata* dominates the number of visits to *Alkanna* flowers. Further studies need to be carried especially on the species that showed significant effects in transferring pollen to the stigma. It would be helpful to know how effective they are in seed production, and how far they can move pollen and hence genes.

2.6 Interesting observations

A few times during the study I noticed *A. pauperata* visiting unopened flowers, especially early in the morning and in the late afternoon, spending a significantly longer time to collect the resources (mostly pollen) of each flower, repeatedly visiting and revisiting the same flower. As in Sinai (Stone *et al.*, 1999), the flowers probably start presenting pollen before they are fully open, and bees were probably harvesting unusually large amounts of pollen by this strategy.

Normally during the day I saw many strikes (contact interactions in the air) between male and female *A. pauperata*. During the time when females were foraging from flowers and males were flying around their territories, a male would fly apparently aggressively (with a different flight sound) near to the female, trying to strike her, while the female tried to avoid being struck by rapid oscillatory flight movements or by flying away to another plant. The male was successful in striking the female a few times each day. Most of these interactions were either very quick and aerial, occurring while females were trying to avoid being struck, or on the flower while the female was foraging. However, I once saw an apparently aggressive interaction in the air where both bees fell down onto the ground together for a few seconds - this may have been a mating. In sometimes, the male flew aggressively near to a female who did not try to avoid or escape, yet the male chose to leave without contact. I also saw the same female struck twice, probably each time by a different male. These interactions could be part of mating, or they could constitute the eviction of unfamiliar females from another territory. If this is mating, then why do males sometimes choose not to strike a particular female? Is it because the female has already been mated? Males might be able to discriminate between mated and unmated females, as in *Amegilla dawsoni* and other

species where such discrimination occurs via pheromonal signals produced by mated females (Thomas, 2011). I saw the same kind of interaction between males and females occurring within *Eucera* (*Synhalonia*) sp. 1 and *Anthophora disparalis*.

Megachile (*Chalicodoma*) sp.1, the second commonest visitor to *A. orientalis* flowers, sits on the ground more frequently than *A. pauperata* and stays there for an obviously longer period of time. It is also a slower forager, spending longer time to collect nectar from a single flower, and thus the visits are much longer in total.

In difficult weather with wind and heavy rain, the bees disappeared. The day after, I saw female *A. pauperata* feeding for obviously longer times from individual flowers of *A. orientalis*, as well as making unusual visits to the flowers of other surrounding plants (*Astragalus spinosus* and *Zilla spinosa*) for both pollen and nectar. These unusual visits might be because of hunger from the previous day, and/or because the flowers were unusually full of uncollected rewards, and/or maybe nectar of *A. orientalis* was diluted by rain. Normally I very rarely saw female *A. pauperata* visiting flowers other than *A. orientalis*.

Sometimes both male and female *A. pauperata* rested on the ground during foraging activity. Even less frequently, but staying for lo -7 min), I saw *A. pauperata* sitting on the top of long grass stems or on a leaf of a nearby tree (usually an olive tree). Perhaps these were longer rests for self-cleaning and excretion.

2.7 Acknowledgements

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Chapter 3: Daily pattern of territoriality in an anthophorid bee: a case study from Jabal El-Loz, northwestern part of Saudi Arabia

3.1 Abstract

Territoriality is often seen as a strategy to increase the number of mates of successful males, but the behaviour of a solitary anthophorid bee (*Anthophora pauperata*) in Sinai has previously been interpreted as investment of resources in offspring. I studied the same system in the northern part of the western highlands of Saudi Arabia to test this idea. Males showed a strong diurnal pattern of territoriality enclosing blooming *Alkanna* flowers. Just as in Sinai, the females foraged for nectar and pollen early and late in the day, while the males performed territory defence in between these times. Female nests were dispersed within their home ranges that contained food resources, and females copulated mostly with territory-holding males. Thus I found little support for the idea of territoriality as paternal resource investment. Females were shown to be able to travel long distances regardless of the harsh landscape and high mountain barriers, and hence can move genes among wadis.

Key words: Territoriality, *Anthophora pauperata*, solitary bee, mating, and foraging.

3.2 Introduction

Territoriality is a strategy utilised by male insects of many species to gain matings (Alcock, 1975; Alcock *et al.*, 1978; Velthuis *et al.*, 1980; Paxton, 2005). Territorial males differ in their behaviour, with some holding defended territories and others patrolling a largely undefended home range. Those with a home range often perform a regular loop by visiting a chain of spots along their flight route, usually with few or no interactions with other males (Alcock *et al.*, 1978); on another hand, those holding territories, 'real territorial males' (Willmer *et al.*, 1994; Gilbert *et al.*, 1999), may have no patrol routes, but mostly defend their territories with aggressive interactions (Willmer *et al.*, 1994; Sugiura, 2008). These territories are set up wherever females occur, at their foraging or nesting areas, or where they can be attracted by scent-marking males (Eickwort *et al.*, 1980; Ayasse *et al.*, 2001). The function of such behaviour is to gain matings, as is usually obvious. It is a puzzle, therefore, when males and females hardly ever meet, and matings are hardly ever seen.

The 20,000 species of bee (Apoidea) are distributed extensively all over the world (Michener, 1974), and many species have been confirmed as showing male territoriality. The size of the territory varies greatly according to the territory characteristics and the density of competing conspecific males (Alcock, 1977; Eickwort, 1977; Eickwort *et al.*, 1980; Willmer *et al.*, 1994). Defended territories are usually among flowers at sites where females are foraging (Eickwort, 1977; Stone, 1990; Willmer *et al.*, 1994; Sugiura, 2008), at female nesting accumulations (Barrows, 1976), or uncommonly at female emergence sites (Alcock *et al.*, 1976; Alcock, 1997). These are all sites at which males can secure matings. The variety of sites occupied, the different patterns of male behaviour, and the range of mating systems are reviewed in Paxton (2005). Male-male

aggressive behaviour can differ among sites: at foraging sites this can involve chasing and fleeting physical contact, whereas at nesting or emergence sites this can end in damaging physical fights.

As important pollinators (Willmer, 2011), the detailed behaviours of both males and females are significant in the context of plant gene flow via pollen, and over and above the behaviour, the structure of the landscape can enhance or restrict gene flow of both bees and plants over a large geographical scale by encouraging or limiting bee movements among sites (Baguette *et al.*, 2013). Gene flow between populations is an essential factor to define the structure and evolution of populations (Burczyk *et al.*, 2004). Large bees often travel long distances and perhaps are therefore more effective agents of plant gene flow in fragmented landscapes (Gathmann *et al.*, 2002; Greenleaf *et al.*, 2007; Zurbuchen *et al.*, 2010). For solitary or specialist (oligolectic) bees, movements are normally expected to be restricted to their territories and home ranges (Zayed *et al.*, 2005), and hence genetic differentiation of bees and plants among sites may occur due to increased inbreeding in isolated populations, eventually leading to an increased risk of extinction. A high risk of extinction has been correlated in many studies to the degree of specialisation (Labandeira *et al.*, 2002; Koh *et al.*, 2004; Zayed *et al.*, 2005). Thus gene flow is important in the conservation of both pollinators and plants, especially for self-incompatible plants.

On the basis of (a) the lack of overlap in diurnal patterns of activity between males and females; and (b) the apparent absence of matings, Willmer *et al*. (1994) made the very unusual suggestion that the function of male territories of the solitary bee *Anthophora pauperata* in the South Sinai mountains was to guard nectar and pollen resources during the day for females to collect in the late afternoon, after male activity

had disappeared. Presumably the males do this in exchange for matings. The problem with this explanation is that it is clearly cheatable (Gilbert *et al*., 1999): females mated by different males can collect the resources, and non-territorial males can obtain matings.

The aims of this study are to try to resolve this issue, by: (1) studying the behaviour of males and females in the same system (*Anthophora pauperata* bees with *Alkanna orientalis* flowers) recently discovered at Jabal El-Loz in Saudi Arabia; and (2) testing whether the bees can move long distances among wadis (valleys). I conclude that the territories probably function to gain matings, as in many other bees; and that long-distance pollen movement occurs via female movements among wadis.

3.3 Materials & methods

The study took place throughout March and April 2015 at Jabal El-Loz (Fig 1: Madiyan mountain range, Tabuk region) in the northern part of the western highlands of Saudi Arabia (N 28º 43.452' E 35º 19.417'). The mountainous topography is similar to that of St Katherine in South Sinai (Egypt), 140 km away across the Gulf of Aqaba, where the same *Alkanna-Anthophora* system has been studied previously (Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Wolff *et al.*, 1997; Stone *et al.*, 1999; Nuttma *et al.*, 2006). It is a barren area with a maximum altitude of 2580 m. The climate is normally excessively dry, with hot rainless summers and cool winters. The mean annual rainfall is very low, with a mean of 20-40 mm in the northwest (Hasanean *et al.*, 2015) but regularly with long periods of drought throughout the year broken by high-intensity rain and floods. The mean annual temperature is about 22.5 °C (Almazroui *et al.*, 2013). The landscape is

overwhelmed by rugged mountains with intervening steep-sided wadis. Seven separate but geographically close wadi systems, each with 1-3 sites separated by more than 500 m, were deliberately chosen to be able to detect small- and large-scale movements (Fig. 3.1): they were labelled by wadi number and plot letters within wadis.

The study was focussed on the solitary anthophorid bee *Anthophora pauperata* Walker (Apidae, Anthophorinae: henceforth referred to as *'Anthophora'* - supplementary S1), the main pollinator of *Alkanna orientalis* (L.) Boiss. (Boraginaceae: henceforth *'Alkanna'*) in the Sinai desert of Egypt (Stone *et al.*, 1999), and also in northwestern Saudi Arabia (this study). The plant is distributed extensively on the mountaintops of the eastern Mediterranean and Middle East regions, with an exceptionally isolated colony in Algeria (Gilbert *et al.*, 1999; Nuttm *et al.*, 2006). In contrast, this species of *Anthophora* had only been recorded from Sinai before this study. Males are smaller, grey-haired and in our study site (although not in Sinai) always with yellow facial markings, while females are larger with a brown-haired abdomen (Willmer *et al.*, 1994).

247 *Anthophora* bees were marked at the start of the study (in early March 2015) using numbered coloured plastic discs (obtained from EW Thorne, Wragby, Lincs., UK). Bees were given a distinguishing colour different for each of the 12 sites. The discs were attached to the thorax (Fig. S2) using a tiny amount of glue (Instant bond gel Su aglue). I used a net to capture each bee, anaesthetising them with carbon dioxide (CO_2: Sparklets dispenser) for attaching the disc. Each bee was carefully allowed to recover and fly away voluntarily.

The size of territories was fairly small, about 5 to 10 m across. Thus a single or pair of observers could easily monitor the patrolling routes of individual males, watching

movements among clusters of *Alkanna* plants over the whole period of bee activity each day. These observations were carried out in three sites (1A, 2B & 3A), where I watched a particular individual territorial male for two days from morning appearance until disappearance in the afternoon (ca. 08:0(– 17:00). Detailed records were taken for ten-minute periods every half-hour. These systematic data on male behaviour were complemented with detailed observations.

In each observation period I noted the number of encounters of the territory holder with other territorial or non-territorial males, as well as with females, clarifying the nature of all encounters where possible. Most often male-male encounters involved chasing, followed by the foreign male leaving and the territory holder returning to his territory. Sometimes aggressive fighting occurred, with one or both males falling to the ground for a few seconds to recover (Fig. S2-A), which happened also in interactions with other species. In male-female encounters, chasing occurred sometimes, while at other times they involved attempted or actual copulation (Fig. S2-C). Attempted copulation included quick approaches from the side with sudden physical interactions avoided if the female flew off; males pursuing copulation hovered near a flower-feeding female and tried to contact her from the back. In actual copulation, the male and the female locked together for a few seconds, commonly falling to the ground: there seemed to be little doubt that this was a mating. I have no data on female-female interactions as it rarely happened: it was sometimes evident in the afternoon when females were starting to collect pollen.

Each territorial male performed a pattern or a variety of patterns of patrol loops which could be monitored. These patterns of patrol loops were two-dimensional and close to the ground, and hence I could easily measure the length of each patrol route. I

also recorded the number of actual visits to flowers made by the territorial male while patrolling.

Female *Anthophora* forage on clumps of *Alkanna* plants located across the territories of several males, and hence theoretically a female could be mated several times by different males; however, nearly all interactions were with territorial males. I have no data on the reproductive success of territorial vs. non-territorial males because by definition the non-territorial males roamed much more widely and it was impossible to keep them under continuous observation: their reproductive success appeared to be very low. Few nesting sites were found: these had entrances under rocks or flower clumps of either *Alkanna* or other plant species, all within the territories of particular males.

The positions of all marked bees were recorded by walking all sites three times during the study period: early on at the beginning, at the middle of the study period, and at the end (Table S1) to record bee movements.

To document the microclimate, the ambient temperature, wind speed and humidity were recorded at regular times during all observation periods, using a Skywatch Atmos Thermo-Hygro-Anemometer. In analysing the behavioural data, to achieve normality the data (for the periods of time when males were sitting on the ground, and the number of visits to flowers) were square-root transformed. Google Earth software was used to measure movements between sites. All mean values are given ± one standard error; all times are in local Saudi time (GMT+

3.4 Results

Anthophora was one of the first bees to appear in the wadis in early spring, and both males and females were present from the beginning of the study early in March. This early appearance was synchronized with the early flowering of *Alkanna*. The numbers of *Anthophora* increased until the 20th of March, when they started to de noticeably again.

During the diurnal activity period, male *Anthophora* conducted repeated patrol loops over particular sets of flower clumps, which identified their territory borders. Each male territory normally contained at least one fairly large plant with more than 100 open flowers, which acted as the main resource visited regularly by the male between his patrols, along with a further number of small plants. Territories enclosed about three to ten plants at the start of the day, progressively increasing over the day as more plants were incorporated into the territory. The territorial male behaved aggressively with most other bees, as well as with flies, dragonflies, wasps or any insect that approached the flowers of his territory. He fed intermittently on flowers, often start sampling many of them in the early morning, usually on particular large plants. As the day progressed, he sampled less and flew more and faster between the plants in his territory.

Figures 2-4 illustrate maps of three territories with the various routes of an individual patrolling male in each territory over two days of intensive observation. These particular males had territories of different size and complexity, and are representative of male movements in large and small territories.

In site 1A (Fig. 3.2), the male showed the same pattern on both . The territory was quite small at about 7 x 4.2 m. He appeared at 08:20 h and performed

various patrol routes. After 10:20 h, he tried to expand the territory by adding two plants about 6 m away, but was chased away by the territorial male whose territory contained those two plants. Both males kept chasing each other sporadically during the day on both days.

In site 2B (Fig. 3.3), the male also showed the same patte on both days. The territory was moderate in size, at about 6 x 8 m. He first appeared at 08:45 h, and performed a simple patrol route around flowering *Alkanna* plants. By doing this he is protecting his territory from another territorial male rather than expanding his own territory.

In site 3A (Fig. 3.4), the male showed a much more complex pattern across quit a large territory of about 18 x 16 m, enclosing more than 24 flowering plants. He appeared at 08:40 h in both days. In the first day, he starts his journey with the first set of plant and later in the day he added the second set of plants. In the second day, he performed the same pattern of patrolling routes on the first day and even later he added a third set of plants. Before the end of his second day's activity he had succeeded in adding a fourth set of two large plants to his territory, after many fights with another territorial male throughout this and the previous day.

To investigate how long the same territorial male kept holding the same territory, ten territorial males (including those of Figs. 2-4) were checked once a day for a week. Two of them continued holding the same territory for four days, and another for three days. Of the males of Fig 2-4, two continued holding the same territory for five days, one performing similar patrol loops for the whole week. Four males disappeared: perhaps their ID tags became dislodged, or they moved location, were killed by

predators, died, or perhaps just failed to continue normal behaviour when the competition became intense.

At their two peaks of foraging in the early morning and again in the afternoon, females just fed from the flowers, typically visiting a large number of the open flowers on each plant, several hundred on a large plant. They foraged peacefully with other species, and showed no aggressive behaviour even with passing males. Many male-female interactions were recorded throughout the male activity period (Fig. 3.5A). The number of interactions was significantly different among hours of the day, with the highest number of interactions in the early morning before 09:00 h. There were significant differences among sites, with the highest number of interactions in site 1 (Fig. 3.5B).

Male-male interactions were recorded throughout the male activity period (Fig. 3.6A). The number of interactions was not significantly different among hours of the day, with many interactions throughout the male activity period. There were significant differences among sites, with the highest number of interactions in site 1A (Fig. 3.6B).

Males were frequently to be seen sitting on the ground during the active period of the day. The periods and the frequency of sitting were highly significantly different among hours of the day (Fig. 3.7A), high in the early morning, then decreasing until the final hour before the males disappeared, when it increased slightly. There were again significant differences among sites, with the lowest frequency of sitting in site 3A (Fig. 3.7B).

Males fed sporadically to acquire the energy to provision their flight activity. The amount of feeding was highly significantly different among hours of the day (Fig. 3.8A),

highest in the early morning, decreasing to a minimum at noon and then rising slightly again before the males disappeared. Males fed more on larger plants (Fig. 3.8B).

Many of the marked bees were not seen again, and few movements were recorded. Only females were recorded as having moved among sites (Fig. 3.9). Some of the movements (n=1) occurred between connected sites within wadis e.g. 3A/3B, and others (n=3) occurred between unconnected sites of different wadis e.g. 1A/3A and 2A/4A. The largest recorded movement was about 3750 m from site 2A to site 4A three weeks later. Another two movements of marked bees were recorded between site 3A and site 1A, a distance of about 2150 m, one over two weeks, and the other one week. One more movement was recorded from site 3B to site 3A three weeks later, a distance of about 650 m.

Many marked male and female bees disappeared (Table S1): either they went to other unmonitored sites, lost their marks, died, or were killed by predators. Of the total number of bees marked at the beginning, 32.4% remained at the same site at the end of the first week, 24.3 % at the end of the second week, and 16.2 % at the end of the third week. There were significant differences in the proportion of bees remaining by the weeks, a sex x week interaction and a site x week interaction (Table 3.1), but no overall site or sex differences. To calculate the percentage of male and female remaining at the same site, I considered the first week after bees had been marked as the origin because of the possibility of marking-induced movement (Fig. 3.10). The percentage of male individuals remaining from the first to the second week was 81.7%, and from the second to the third 75%. The equivalent numbers for females were 94.5% and 49.6% respectively.

Table 3.1: ANOVA with binomial errors of the proportion of marked *Anthophora pauperata* bees remaining at the same site through time, as predicted by site, sex, and week. The test was done by sequential deletion, tested by chi-squared.

Effect	*Df*	X^2	*P*
site : week	13	26.6	0.013
site : sex	12	15.8	0.2
week : sex	3	11.6	0.009
site	11	15.6	0.2
week	2	11	0.004
sex	1	0.6	0.4



Figure 3.1: Study plots in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The red pegs show the plots studied for male territorial behaviour. Both the yellow and red pegs show plots used for studying bee movements. The white lines show the path of the wadi beds. The highest elevation was site 1A, and the lowest site 7A.

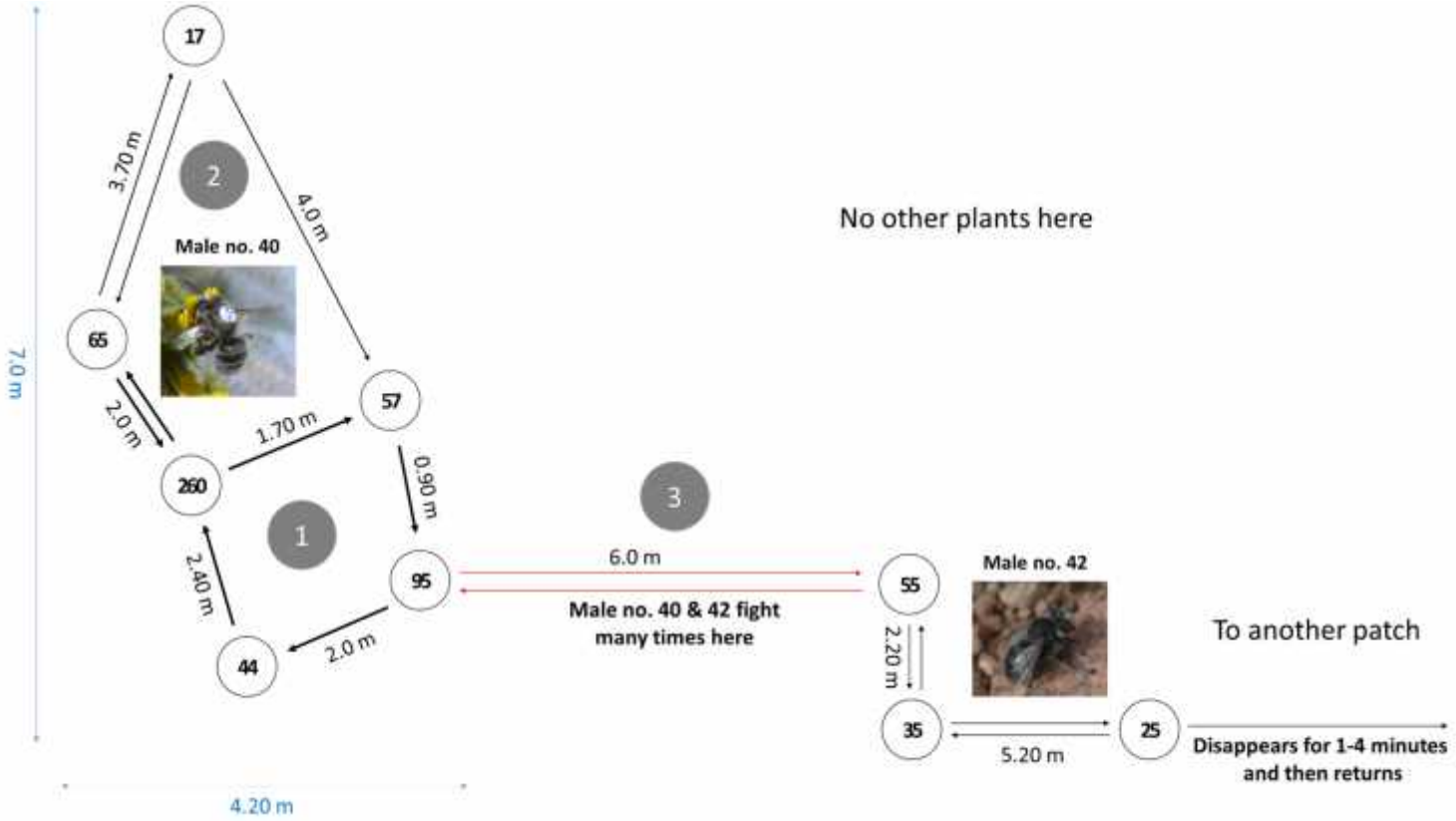


Figure 3.2: Map from site 1A at Jabal Al-Loz on March 2018, showing the patrolling routes of the observed male *Anthophora pauperata* (#40) and his neighbour (#42). Male #40 used three types of loops around six *Alkanna orientalis* plants (mainly in loop (1) of five plants) of different sizes containing 538 flowers in total. The three patches of his neighbour #42 and his movements are also shown. Circles represent plants, labelled with the total number of open flowers.

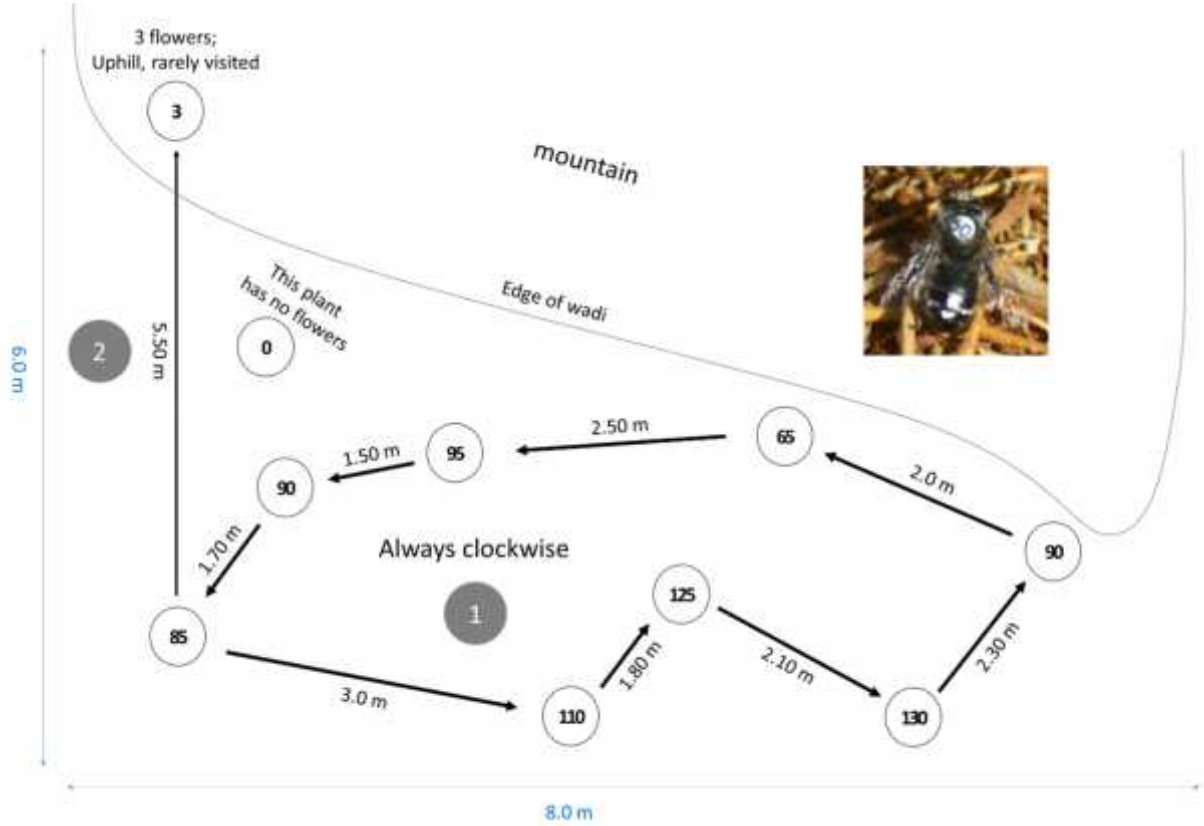


Figure 3.3: Map from site 2B at Jabal Al-Loz on March 2018, showing the patrolling routes of a male *Anthophora pauperata* using two types of loops (1 and 2) around 9 *Alkanna orientalis* plants of different sizes containing 793 flowers in total. He started his patrolling route in the early morning with the first loop (most performed loop), and later in the day he added one further plant and performed the two types of loops. Circles represent plants, labelled with the number of total open flowers.

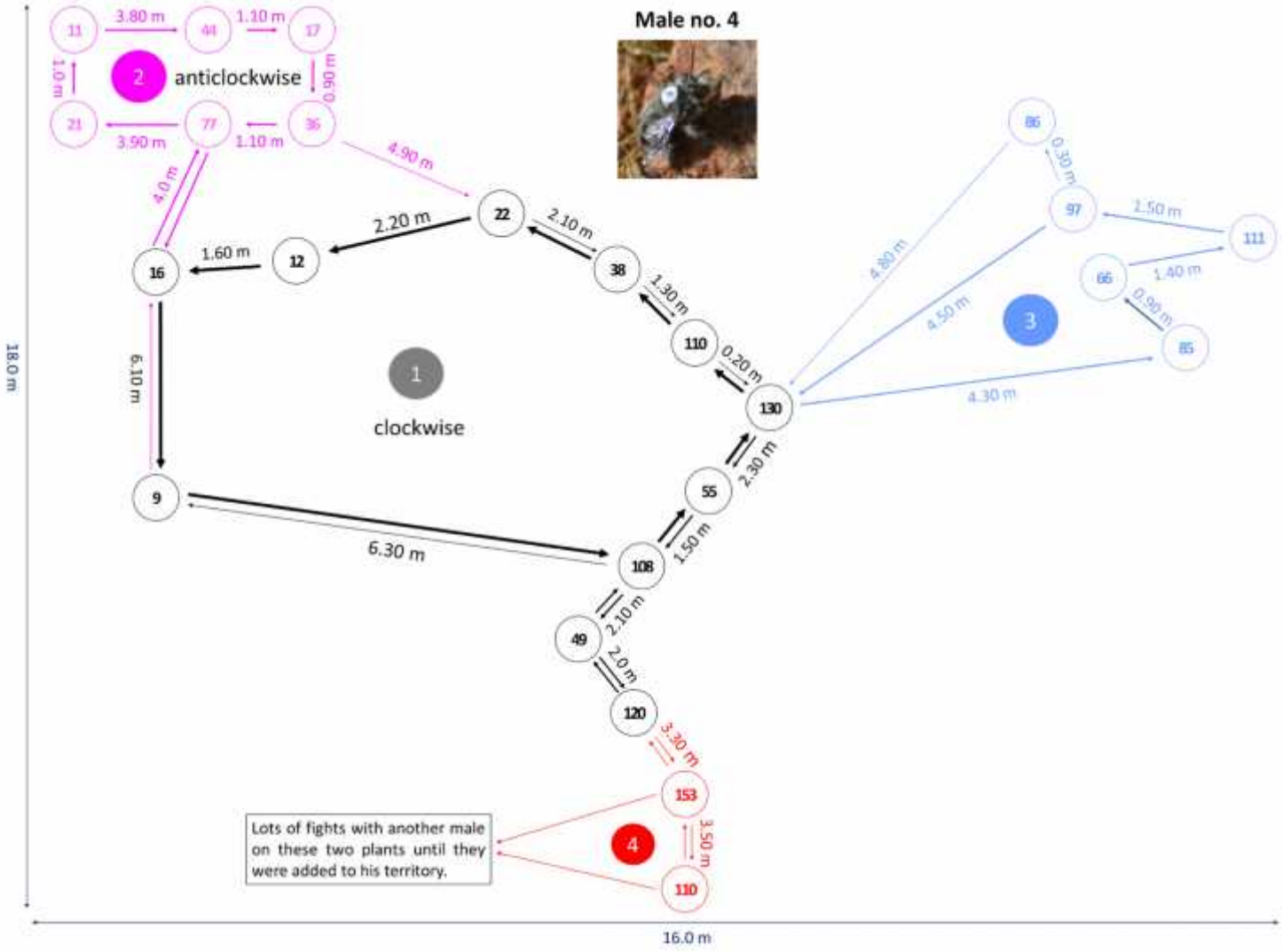


Figure 3.4: Map from site 3A at Jabal Al-Loz on March 2018 shows the patrolling routes of a particular male *Anthophora pauperata* showing different types of loops around 24 *Alkanna orientalis* plants of different sizes containing 1320 flowers in total. In the early morning, the patrolling male showed three types of loops in a first set of 11 plants (1) (black routes). Later on, he added a second set of six plants and showed two types of loops around them (2) (pink routes). Even later on the second day, he added a third set of five plants and showed two types of loops around them (3) (blue routes). Before the end of the second day of observation, he added a fourth set of two large plants to his territory (4) (red routes). Circles represent plants, labelled with the number of total open flowers.

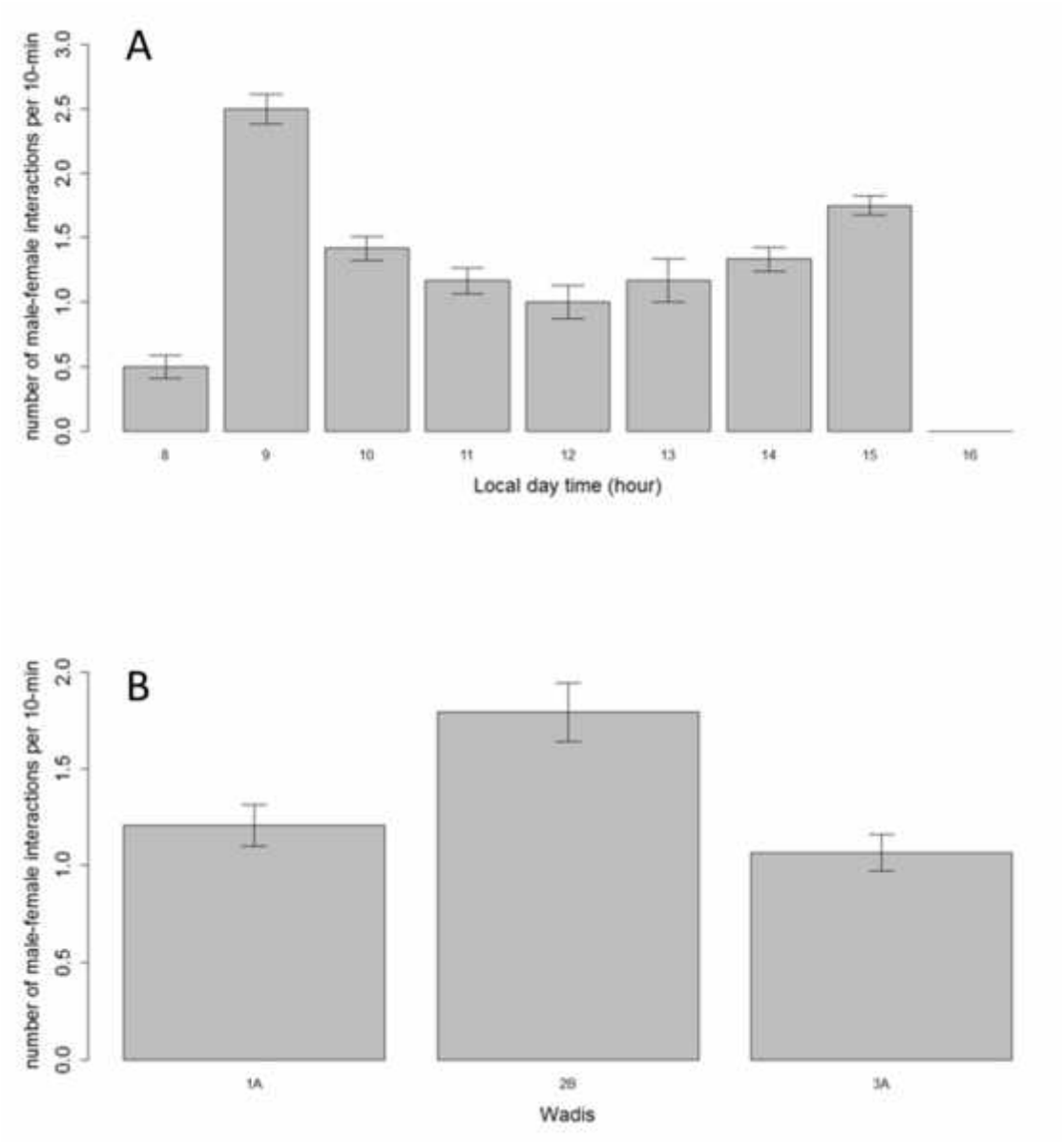


Figure 3.5: (A) Mean (± se) number of male-female interactions of *Anthophora pauperata* per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are significant ($F_{8, 80}$ = 3.221, P = 0.003). (B) Mean (± se) number of male-female interactions of *Anthophora pauperata* per 10-min observation period among three wadis. The differences among means are significant ($F_{2, 86}$ = 3.4, P = 0.03), all accounted for by the higher value of wadi 2B.

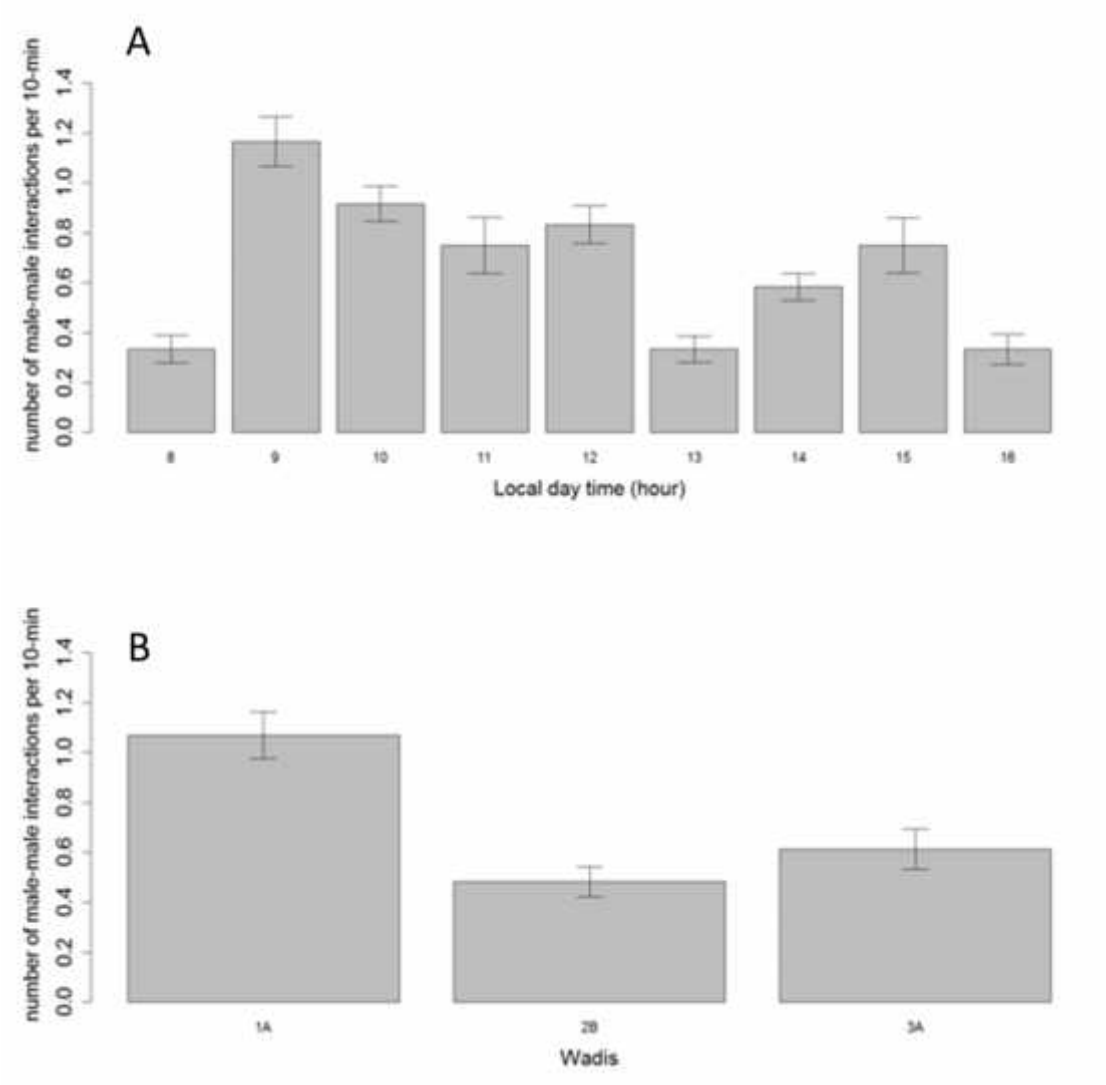


Figure 3.6: (A) Mean (± se) number of male-male interactions of *Anthophora pauperata* per 10-min observation period along the day local time across three different sites during March-April 2015. Despite the appearance of especially 0800 vs 0900, overall the differences among means are not significant ($F_{8, 80}$ = 1.344, P = 0.23). (B) Mean (± se) number of male-male interactions of *Anthophora pauperata* per 10-min observation period among three wadis. The differences among means are significant ($F_{2, 86}$ = 4.898, P = 0.009), all accounted for by the higher value of wadi 1A.

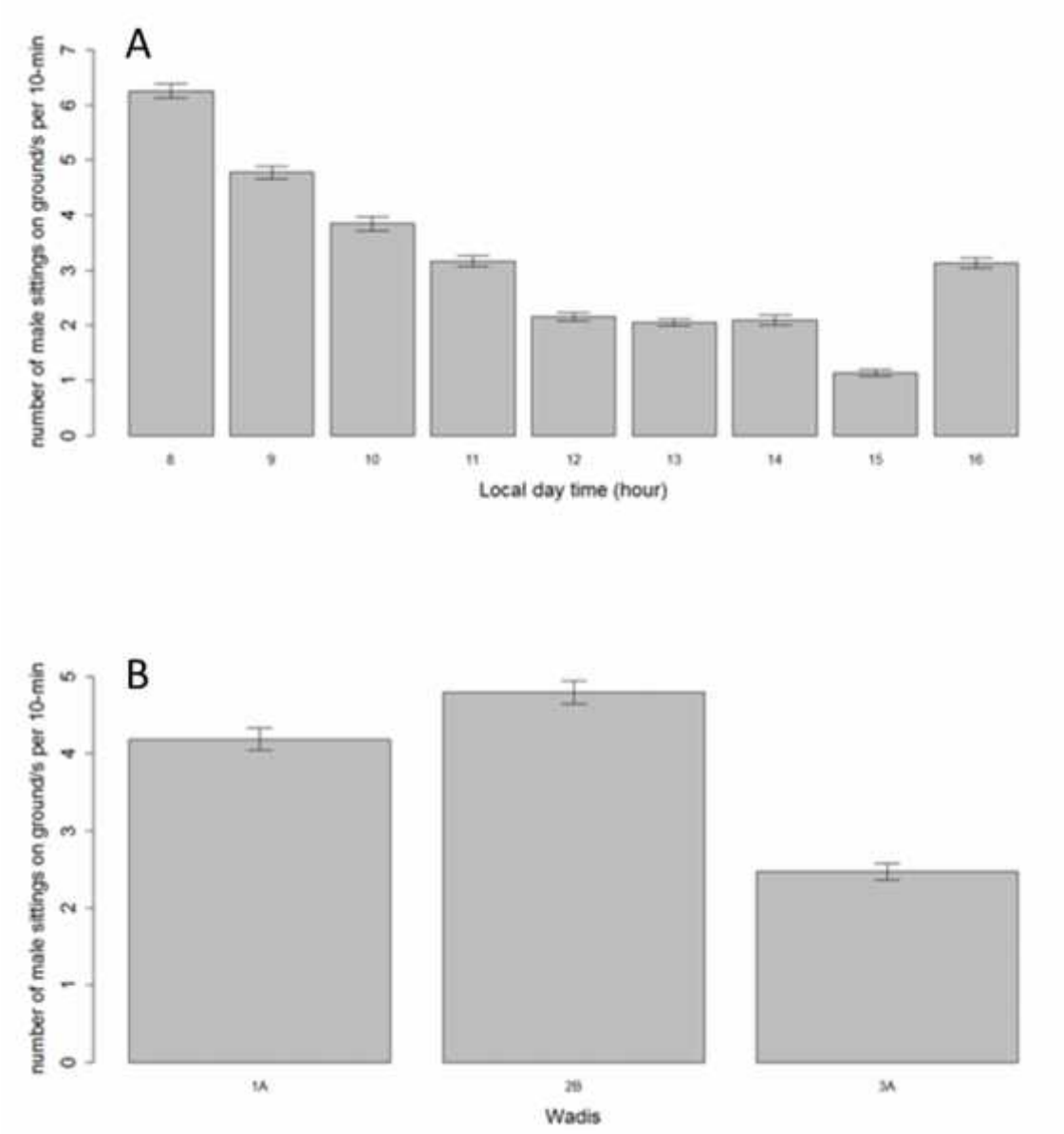


Figure 3.7: (A) Mean (± se) number of *Anthophora pauperata* male sittings on the ground per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are highly significant ($F_{8, 217}$ = 25.34, P << 0.001). (B) Mean (± se) number of *Anthophora pauperata* male sittings on the ground per 10-min observation period among three wadis. The differences among means are highly significant ($F_{2, 223}$ = 23.98, P << 0.001), all accounted for by the lower value of wadi 3A.

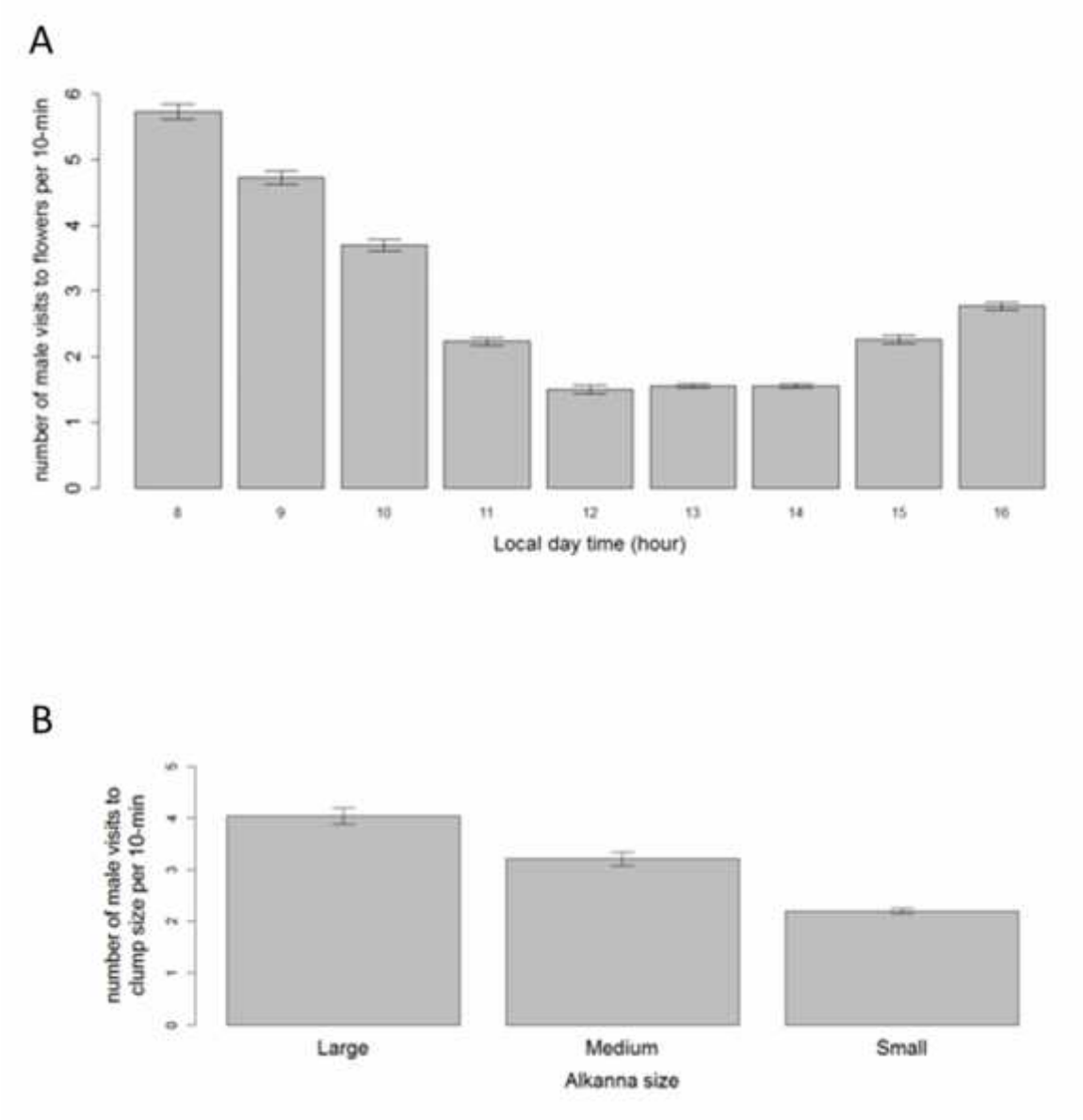


Figure 3.8: (A) Mean (± se) number of visits of *Anthophora pauperata* male to flowers of *Alkanna orientalis* per 10-min observation period along the day local time across three different sites during March-April 2015. The differences among means are highly significant ($F_{8,\ 170}$ = 44.82, P << 0.001). (B) Mean (± se) number of visits of male *Anthophora pauperata* to different clump sizes of *Alkanna orientalis* flowers per 10-min observation period throughout the day across three different sites during March-April 2015. The differences among means are significant ($F_{2,\ 174}$ = 8.54, P = 0.0002).

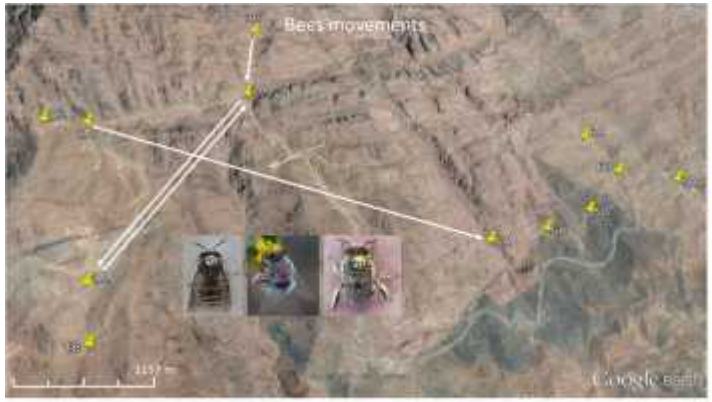


Figure 3.9: The movements of female *Anthophora pauperata* between sites at Jabal El-Loz in the northern part of the western highlands of Saudi Arabia during March-April 2015.

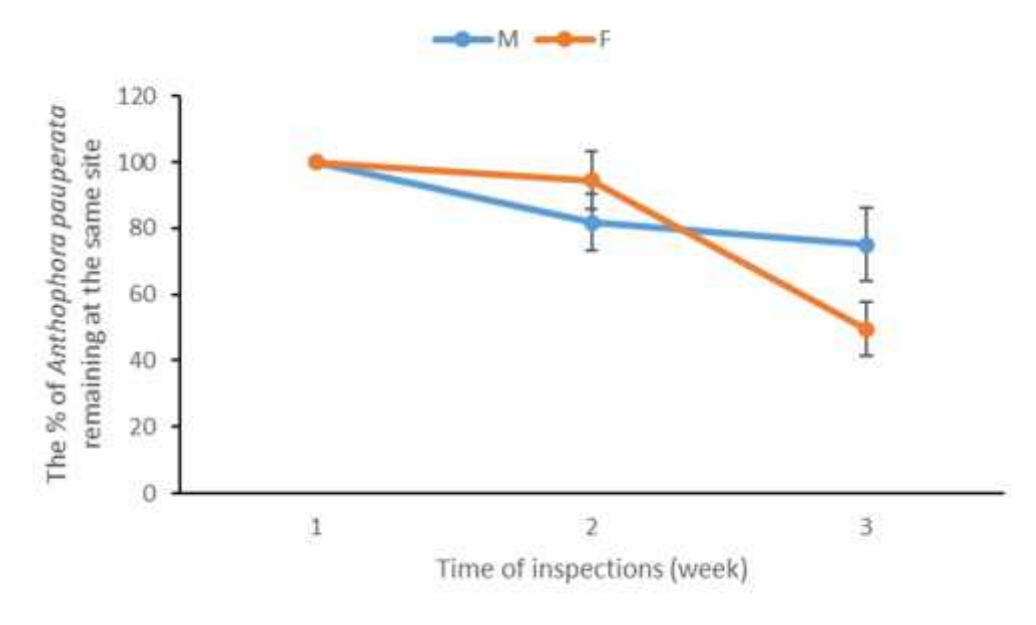


Figure 3.10: Mean (± s.e.) percentage of marked male and female *Anthophora pauperata* remaining in the same site through time (weeks) across twelve different sites during March-April 2015.

3.5 Discussion

This study is comparable with those conducted in Sinai more than twenty years ago on the same bee-flower system (Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Wolff *et al.*, 1997; Stone *et al.*, 1999; Nuttma *et al.*, 2006), and the behaviour is very similar. *Anthophora pauperata* appeared early in March in northwestern Saudi Arabia, earlier than in South Sinai (the 20th of March: Zalat, 1984; Willmer *et al.*, 1994). Both males and females were closely associated with a single plant, *Alkanna orientalis*. Female foraging was bimodal, and males arrived in the morning just as females were about to disappear, and left just as females returned to the flowers in the late afternoon. Thus the same peculiar separation between males and females was evident in Saudi Arabia. Males held clearly defined territories, and showed various types of behaviour through the day: patrolling, foraging, resting, mating interactions with females, and aggressive interactions with other territorial males and some other species. The aggressive behaviour was more intensive at the time when females were away (presumably attending to their nests).

Although most of the time females foraged peacefully next to others, female-female interactions sometimes were obviously aggressive, just as recorded in Sinai. Gilbert *et al.* (1999) interpreted this as either a group of females associated with a particular male, or perhaps related and sharing same resources for the period of nesting and foraging. These female-female interactions occurred mostly in the afternoon foraging period, at the time of pollen collection, and could represent a level of competition for pollen, probably a limiting resource.

Willmer *et al.* (1994) interpreted the apparently mutually exclusive diurnal activity patterns of the sexes as an attempt by males to preserve food that females will gather later on in the day to provision a new cell. Thus, they argued, male behaviour

was not merely to secure matings: it resulted in an investment in their offspring. This is a puzzle because of the obvious cheatability of such a system (Gilbert *et al.*, 1999): why should males bother to be territorial when they can just secure matings without it? In Saudi many matings were obtained by non-territorial males, and in one case I saw a non-territorial male mate a female and then the territory holder evict him and then return to mate the same female. Thus the crucial feature is the mating behaviour of females (Gilbert *et al.*, 1999). If females are receptive to mating only once, or on a few defined occasions (such as when they start a new nest), then the territoriality of the males could be a strategy to catch new unmated females, or perhaps also receptive nest-searching females. This would explain why (a) males are territorial when foraging females seem to be largely but not completely absent; and (b) both the Sinai and Saudi studies repeatedly noted that territorial males fly around some females and leave them untouched. In this study, territorial males sometimes settled for relatively long periods on grasses or tree leaves, perhaps a type of inspection for emergence sites.

In territorial bees of many species, males are scarce compared to females (Alcock *et al.*, 1978). In our case of *Anthophora*, both sexes occur at the site of the flowers, and males are present at high density, holding adjoining territories that enclose most of the existing plants. Females forage within male territories, and as in many other solitary bees (*Anthidium manicatum*: Severinghaus *et al.*, 1981; *Calliopsis persimilis*: Danforth, 1990) they can mate more than once, possibly once for each egg laid per day per each female (Willmer *et al.*, 1994) but the rarity of obvious matings militates against this interpretation. In many bees, polyandry is a common strategy, whilst in other species the females mate only once at emergence (reviewed in: Paxton, 2005). Polyandry is the implication from the little that is known about the Anthophorinae (*A. occidentalis*: Batra,

1978; *A. plumipes*: Stone, 1995). At Jabal El-Loz, as in Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1999), matings could have occurred every day in the morning in the short period when males and females coincided in the wadis, although this was definitely not obvious. If true, each egg laid could potentially be fertilised by sperm from a different male, although usually each territory holder encountered the same females and attained most matings with them. Thus most females would be mated frequently by the same males holding the territories that overlap with their foraging home ranges, and occasionally by sneaker males. She may mate multiply to increase the genetic quality of her offspring, or to avoid the cost of resisting further matings.

There are also common features of the behaviour of *Anthophora pauperata* with other territorial bees. *Anthophora pauperata* at Jabal El-Loz showed different mating strategies, as in Sinai (Willmer *et al.*, 1994) and many other territorial bees (Stern, 1991; Starks *et al.*, 1999; Leys *et al.*, 2008; Oliveira *et al.*, 2010). Perhaps when there are few territorial males and/or to avoid the cost of holding territories, some males range widely, invading the territories of many other males, and encountering and mating with females in the short period before being noticed and chased away by the territory holder. The existence of such 'sneaky' males is common in many insect genera, producing at least two alternative strategies (Severinghaus *et al.*, 1981; Gross, 1996). In some circumstances, these strategies are associated with male physical features, but this does not seem to apply to *Anthophora pauperata*, where there is no obvious visible difference between territorial and non-territorial males.

It is worth emphasising the contingent nature of the territorial system in *Anthophora pauperata*. Foremost, male territoriality depends upon the density of females. In our study in early March, in one site territorial behaviour did not start for a

week after observations started, when the number of females had noticeably increased. The 100 x 100-m site enclosed only 19 scatter *Alkanna* plants, and so probably the costs of territoriality made it unfeasible at the beginning of the season. In this site, males were much commoner than females, whereas in all other sites the reverse was true. In some more open sites overall bee densities were low, and here too there was no obvious territoriality. I interpret these data as suggesting that territoriality only occurs where flower, bee and female densities are high enough.

There was a significant difference among sites in the number of male-female interactions and in the time males spent sitting on the ground. One site had the lowest of the former and the highest of the latter, probably because the low density of territories meant that the male fought only with passing other males, and not with neighbouring territory holders. Thus the holder male was much relaxed in defending his territory and spent more time in searching for females for matings. Interestingly, while the holder male sat on the ground, he would move instantly when a stranger or especially intruder *Anthophora* male arrived in the territory, otherwise spending much longer on the ground than in other sites.

In many bee species, the duration of a male holding the same territory depends on the level of competition (Eickwort *et al.*, 1980). In some situations rapid changes among individual males, and lots of encroachment can occur. At Jabal El-Loz, male *Anthophora* held the same territories for a number of consecutive days, and thus did not appear to depend on the level of competition. In Sinai one particular male defended the same territory for 18 days (Gilbert *et al.*, 1999). Many territorial male bees are known to use pheromone scent-marking to define their territories (Free, 1971; Kullenberg *et al.*, 1973; Tengö *et al.*, 1976; Kimsey, 1980; Marshall *et al.*, 1981; Kroiss *et*

al., 2010), but there was no evidence this at Jabal El-Loz or in Sinai (Willmer *et al.*, 1994). Since males return back repeatedly to the same set of plants over many days, this behaviour suggests that the male sleeps overnight somewhere in or near his territory. Males did not seem to have travelled in the early morning, appearing suddenly to warm up at the site via frequent long periods of sitting.

In this study movements of female *Anthophora pauperata* were recorded between wadis and sites at Jabal El-Loz. The largest movement between unconnected sites was about 3750 m, but this was measured in a straight line and hence the real route is expected to be much longer. Interestingly, therefore, this is proof that female *Anthophora* can travel long distances regardless of the harsh landscape and high mountain barriers, and hence can provide genetic connectivity between wadis. The only previous study in South Sinai (Wolff *et al.*, 1997) suggested gene flow mainly via seeds during flash-flooding events rather than via pollen movement mediated by bees, which was regarded as unlikely because of their territorial behaviour and the high mountain ridges separating the wadis militating against movement among wadis. However, this study shows this is wrong: bees can move pollen long distances. Many other studies confirmed the ability of solitary bees to move pollen long distances. Janzen (1971) found that euglossine bees of lowland wet forest in South America can forage for as far as 23 km away from the nest, visiting the same plants along a standard route, and probably moving pollen long distances. Pasquet *et al.* (2008) used radio-tracking to show that carpenter bees in coastal Kenya were able to forage up to 6 km from their nests mediating gene flow between cowpea flowers. Such long-distance inter-plant movements of solitary bees has been confirmed in a number of species (reviewed in

Zurbuchen *et al.* (2010), but concern just a few individuals among many who remain much more locally.

I conclude that the *Anthophora* sp. at Jabal Al-Loz exhibited very similar behaviour to that in Sinai, apart from its first appearance at the start of the season, which is earlier in Saudi Arabia, and the long-distance movements between separated patches, where it is of wider extent in Saudi Arabia. The genetic study (in chapter 5) alsc supports this finding because the genetic diversity of the Sinai population is much higher than the Saudi population. Such higher genetic diversity of the Sinai population is probably due to the more limited movement caused by external factors. This is discussed in more detail in chapter 5.

3.6 Acknowledgements

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Chapter 4: Phylogenetic and phylogeographic analysis of *Alkanna orientalis* (Boraginaceae) in the Middle East

4.1 Abstract

The short-lived perennial plant *Alkanna orientalis* in Saudi Arabia and Sinai is distributed amongst mountain wadis (valleys) at high elevations. Populations occur both in steep-sided wadis and central plain areas, both naturally isolated by high mountainous barriers. Here I investigate genetic variation within and amongst populations of this plant at different spatial scales using biparentally inherited, nuclear ribosomal DNA (ITS region) and maternally inherited, chloroplast DNA (trnH-psbA region). I find evidence of spatial structure across large geographical scales, which might be the result of historical processes such as range expansion and contraction over long time scales following climatic changes. Notwithstanding such large-scale patterns, there is also evidence of spatial heterogeneity at smaller scales, which may represent more recent, localised events such as population extinction and recolonization from neighbouring areas, or changes in population size coupled with random genetic drift when population size is small. An apparent difference between the distribution of variation in maternally inherited *vs.* biparentally inherited genes could reflect differences in rate of gene flow of pollen *vs.* seeds, or it could be the result of differences in overall levels of genetic variability at the two chosen loci.

Key words: *Alkanna orientalis*, genetic variation, phylogeography, conservation

4.2 Introduction

The global climate has oscillated massively over geological timescales, and notably throughout the Pleistocene through repeated ice ages that have expanded and contracted available habitat. The consequence for most species is altered distributions. In combination with selection driven by local habitat, this results in the genetic structure of populations not being randomly organised, but spatially structured (Allard, 1975; Linhart *et al.*, 1981), i.e. the structure is the consequence of combined factors of migration, selection, and random genetic drift over the previous millennia. In the case of plants such as *Alkanna orientalis*, ecological traits such as the pattern of pollination and the mating system are important in determining genetic diversity and population genetic structure (Loveless *et al.*, 1984).

The level of genetic variation within and among populations varies amongst plant species. Predominantly, high variation among populations is exhibited in self-pollinating species, whereas low genetic variation is exhibited in wind-pollinated species and moderate variation is exhibited in insect-pollinated species (Govindaraju, 1988). These different levels of variation reflect the likely spatial scale of gene dispersal among populations, which is often extensive in pollen movement mediated by insect pollinators (Petit *et al.*, 2005). In these cases, distances moved by pollen will be significantly influenced by pollinator flight behaviour. There are many studies confirming both short and long flight distances of different types of pollinator (Janzen, 1971; Pasquet *et al.*, 2008; Zurbuchen *et al.*, 2010). Theoretica , short flight distances between plants should result in less gene flow and a greater degree of genetic substructure of populations. Notwithstanding the recognised significance of pollinator behaviour on genetic population structure, there are few studies of both the behaviour of known

pollinators and the genetic structure of a particular plant species (Wolff *et al.*, 1997; Van Rossum *et al.*, 2006).

A previous study (Wolff *et al.*, 1997) used RAPDs to analyse population subdivision in *Alkanna orientalis* (L.) Boiss (Boraginaceae) in south Sinai, Egypt. In this study I have extended the studied region and investigated more widely the phylogeographic structure of *Alkanna orientalis* from the Eastern Mediterranean and Middle East. Furthermore, I also estimate the relative amounts of genetic exchange mediated by pollen and seeds, through comparing the genetic structure based on nrDNA (biparentally) and cpDNA (maternally) inherited genes. I selected the trnH-psbA region, a non-coding intergenic spacer in the chloroplast genome that is known to be maternally inherited in angiosperms, and has been used previously to distinguish between closely related species (Ren *et al.*, 2010). I also used the ITS, a non-coding region in the nrDNA known to be bipaternally inherited, which has been used widely for interspecific phylogenetic relationships in similar plant species (Kay *et al.*, 2006).

4.3 Materials and methods

4.3.1 Study area

The study was focused on the eastern Mediterranean and Middle East regions. The eastern Mediterranean region includes Greece, Turkey and Iran; the Middle East region extends this to include Syria, Jordan, Sinai, western Saudi Arabia and Yemen. Fieldwork took place throughout June 2012 in St. Catherine, Sinai, Egypt and during March 2013 at the following locations in Saudi Arabia: three in the northern part of the western highlands of Saudi Arabia, namely Jabal Al-Loz (N 28º 43.452' E 35º 19.417') and Jabal Ash-Shyati (N 28º 13.50' E 35º 34.4 in the Madiyan mountain range of the Tabuk

region, and Jabal Al-Ward (N 26º 24.49' E 37º 13.49') in the Al-Hejaz mountain range of the Madinah region; and three in the Al-Sarawat mountain range of the Asir region in the southern part of the western highlands of Saudi Arabia, namely Jabal Mana'a (N 18º 58.09' E 42º 11.02'), Jabal Nahran (N 18º 10.13' E 42º 27.02') and Jabal Al-Sawda (N 18º 20.21' E 42º 20.37'). All locations included areas of high mountains with both narrow and wide open wadis. Field surveys were in each case initiated 1200 m above sea level on selected mountains, climbing until the summit was reached. Within each wadi, patches of approximately 100 x 100 m were surveyed at intervals of 200-300 m.

4.3.2 Study system

I studied *Alkanna orientalis* (L.) Boiss. (Boraginaceae), a medicinally significant perennial herb with yellow flowers, glandular-sticky leaves, shrubby base and a coloured root that grows in dry places (Tutin *et al*., 1972). It is distributed extensively on the mountaintops of the eastern Mediterranean and Middle East regions, with an exceptionally isolated colony in Algeria (Chapter 2, Fig. 2.4) (Gilbert *et al*., 1999; Nuttman *et al*., 2006). In Sinai, *A. orientalis* grows at elevations higher than 1500 m above sea level (Gilbert *et al*., 1996), and similarly in Saudi Arabia. The plant is thought to reproduce exclusively via seeds and is an obligate outcrossing species (Gilbert *et al*., 1996).

4.3.3 Sampling

A total of 270 individual *Alkanna orientalis* plants were sampled, preferentially selecting young leaves and preserving them in silica gel. 90 individuals were sampled from five different wadis in south Sinai (Arbaien, Shraj, Dir, Itlah, and Farsh Ilia). 120 were sampled from different wadis at Jabal Al-Loz, Jabal Al-Ward and Jabal Ash-Shyati in northern Saudi Arabia, and 60 from wadis in Jabal Mana'a and Jabal Al-Sawda in southwestern Saudi Arabia. A further 28 samples of herbarium material were received from the Royal

Botanic Garden, Edinburgh, UK (14 from Turkey, 2 from Greece, 2 from Syria, 3 from Iran, 3 from Yemen, 3 from southwestern Saudi Arabia, 1 from Jordan), and 6 DNA samples were received from the Royal Botanic Garden, Kew (3 from Turkey, 3 from Greece). All individual plant samples represent different geographic locations with only one plant per location.

4.3.4 DNA extraction and purification

Dried plant leaves were stored at room temperature before DNA extraction. Approximately 30 mg of leaves were ground using a mortar and pestle, followed by DNA extraction using a Sigma-Aldrich DNeasy kit, which uses a selective column to bind DNA preferentially prior to elution. DNA samples were then further purified by ethanol precipitation: 90 µ sterilised water, and 250 µl 100 % ethanol were added to 10 µl of DNA sample, and the solution placed on ice for 20 minutes and then centrifuged for 2 minutes at 14,000 rpm. The flow-through liquid was discarded and the tube kept at room temperature until totally dry; then the DNA pellet was resuspended in 20 µ sterilised water. DNA samples were stored at −20°C and diluted 1/100 if the first attempts to use the solution as a template for PCR were unsuccessful. Despite this extra purification step and further dilution, many samples were still not amplifiable, likely due to poor template quality and/or the presence of impurities in the sample. DNA could be amplified from a total of approximately 53 % (143 out of 270) of the collected leaf samples, about 66 % (4 out of 6) of the DNA samples, and about 85 % (24 out of 28) of the herbarium material.

4.3.5 Genetic markers

To examine the genetic diversity and structure among *Alkanna* populations, approximately 250-bp of a non-encoding sequence of chloroplast DNA, trnH-psbA, was

amplified using published primers (see Table 4.1) (Shaw *et al.*, 2005; De Mat *et al.*, 2011). In addition, an approximately 600-bp section of nuclear ribosomal DNA sequence from the internal transcribed spacer (ITS) was amplified using published primers (see Table 4.1) (Wen *et al.*, 1996).

The polymerase chain reaction (PCR) for the two loci was carried out in a total volume of 30 µl for all specimens utilising a Reddymix™ master mix for the PCR reaction (supplied at 2x concentration for dilution). This total volume consisted of 3 µl DNA used as a template for the PCR in a final $MgCl_2$ concentration of 2.5 mM. Annea temperatures between 50°C and 60°C were tested for each primer in order to establish the most suitable reaction conditions. PCR cycles for the cpDNA (trnH-psbA region) involved an initial denaturation step at 94°C for 7 min, followed by 35 cycles, each comprising of denaturation at 94°C for 45 sec, annealing at 53°C for 30 sec, extension at 72°C for 60 sec and followed by a final extension of 72°C for 7 min 30 sec. The PCR cycles for the nrDNA (ITS region) involved an initial denaturation step at 95°C for 1! followed by 35 cycles, each comprising of denaturation at 94°C for 30 sec, annealing a 58°C for 90 sec, extension at 72°C for 30 sec and followed by a final extension of 60°C for 30 min.

PCR products were separated on 1.5% agarose gel containing ethidium bromide and visualised under ultraviolet light to indicate if amplification had been successful. Successfully amplified PCR products were sent to the Macrogen Company for sequencing. BioEdit and CodonCode Aligner were used to identify variation amongst sequences, and BLAST searching (Camacho, 2013) was used to identify sequences from other closely-related species that have already been deposited in Genbank (Altschul *et al.*, 1997).

Haplotypes were constructed from DNA sequences and genetic differentiation among populations was calculated using the software DnaSP v (Librado *et al.*, 2009). Gene diversities were calculated for each population using the standard formula:

$$(n/[n-1])(1-\sum_{i}^{k} P_i^2)$$

where n constitutes sample size, k the number of different haplotypes and P_i the frequency of haplotype i. Phylogenetic Network software (Bandelt *et al*., 1999) was used to generate evolutionary trees and networks. Phylogenetic trees were constructed using the phylogenetic and molecular evolutionary analyses implemented in MEGA version 4 (Tamura *et al.*, 2007).

4.4 Results

4.4.1 nrDNA: ITS

A total of 160 individuals of *A. orientalis* were sequenced for the nrDNA (ITS region). 13 polymorphic sites (SNPs) were identified (see Table 4.2). A total of six haplotypes were defined, whose geographic distribution is shown in Figure 4.1. Interestingly, five of them were private, i.e. restricted to a single population within the region studied. Measures of genetic variation and the number of private haplotypes observed within each population are shown in Table 4.3. Genetic differentiation among populations was estimated as F_{st} = 0.31 (significance estimated by permutation, 10,000 replica P<<0.0001).

Haplotypes are distributed in a spatially structured manner. H1 dominated the data set (94%), shared in all populatior except those from Turkey. One haplotype (H4)

dominated the Turkish populations, with one individual considered as a sub-haplotype (H5). The other four haplotypes (H2, H3, H5 and H6) were very scarce, detected in just one individual each in a different population.

To explore the population genetic structure of *Alkanna orientalis* further, I investigated the topology of the median joining (MJ) network generated from the six haplotypes (Fig. 4.2). The network clusters the six haplotypes into one main haplogroup where all haplotypes are derived from and separated from their closest relative by one or two mutational steps.

Two outgroup species, *Alkanna tinctoria* and *Alkanna sieberi,* were selected based on their identification using BLAST searching as the closest relatives to *A. orientalis* for which there are sequence data available on Genbank. These were used for the construction of a phylogeny based on the ITS sequence data (Fig. 4.3). On the basis of this phylogeny, haplotypes H1 and H2 appear to be descended from the basal ancestral lineage from which the other types have evolved.

4.4.2 cpDNA: trnH-psbA

A total of 180 individuals of *A. orientalis* were sequenced for the cpDNA (trnH-psbA region). The data set of *A. orientalis* contained 76 SNPs (Table 4.4). A total of thirteen haplotypes were defined, whose geographic distribution is shown in Figure 4.4. The majority of these (nine) were private haplotypes restricted to a single site in the study. Interestingly, six of them were only found in southwest Saudi Arabia. Measures of genetic variation and the proportion of private haplotypes observed are given in Table

4.5. The genetic differentiation among po was estimated as F_{st} = 0.96 (significance estimated by permutation, 10,000 replicate P<<0.0001).

Haplotypes are distributed in a spatially structured manner, where six of them dominated the data set, with the remaining seven being very scarce, found in just one or two individuals. A MJ network based upon the 13 chloroplast haplotype sequences (Fig. 4.5) shows two main haplotype groups separating all individuals from southwestern Saudi Arabia (group B) from all other regions (group A). The network yielded a genealogy where all haplotypes from each main haplogroup are separated from their closest relative by one or two mutational steps. The network topology also separated the two haplogroups by many mutational steps. One clade in haplogroup A was dominated by H3, and six other related haplotypes (H3-derived), while two other clades in haplogroup B were dominated by H6 and H8, each with two groups of rare haplotypes (H6-derived and H8-derived).

The genetic division of haplotypes was substantially congruent with their geographical location. Common haplotypes (H3) and (H6 were regionally separated, with H6 and H8 found in southwest Saudi Arabia, and H3 along the eastern Mediterranean in Sinai, northern Saudi Arabia and Yemen. The number of private haplotypes in haplogroup B (from southwest Saudi Arabia) was higher than that in haplogroup A.

Sequences for related species were selected from GenBank based on a BLAST search to build a phylogenetic tree and to construct a network (Fig. 4.5). The main group of haplotypes (A) was separated from *Alkanna tinctoria* by seven mutations. On the basis

of this phylogeny, haplotype H4 of *Alkanna orientalis* from haplogroup A appears to be from the basal ancestral lineage from which other types have evolved.

Genetic variation among individuals from different wadis within a population was estimated in south Sinai and Jabal Al-Loz. Two haplotypes were identified in south Sinai (Fig. 4.6) and four at Jabal Al-Loz (Fig. 4.7), and individuals from a single wadi shared a mixture of haplotypes in both sites. The genetic differentiation among subpopulations of south Sinai was estimated as F_{st} = 0.2 (significance estimated by permutation, 10,0(replicates, P=0.007); whereas at Jabal Al-Loz was estimated as F_{st} = 0.6 (significance estimated by permutation, 10,000 replicate P=0.0003).

Table 4.1: Primer sequences of the nuclear and chloroplast DNA.

Locus	Position	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size range
ITS	Nuclear (nrDNA)	GTT TCT TTT CCT CCG CT	AGG AGA AGT CGT AAC AAG	550-650
trnH-psbA	Chloroplast (cpDNA)	GTT ATG CAT GAA CGT AAT GCT C	CGC GCA TGG TGG ATT CAC AAT CC	250-300

Table 4.2: Summary of SNPs that generate different haplotypes of *Alkanna orientalis* based on nuclear ribosomal DNA (ITS region) across eight countries of the Middle East and eastern Mediterranean.

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	N
H1	T	A	C	A	C	G	C	A	T	T	T	G	A	150
H2	T	A	C	A	C	A	C	A	T	T	T	G	A	1
H3	T	G	C	A	C	G	C	A	T	A	A	A	G	1
H4	G	A	C	A	C	G	C	A	A	T	T	G	A	6
H5	G	A	C	C	C	G	C	A	A	T	T	G	A	1
H6	G	A	T	A	A	G	A	G	T	T	T	G	A	1

Table 4.3: Summary statistics for nuclear ribosomal DNA haplotypes (ITS region).

Population	N	Private haplotypes %	Gene diversity
Northwestern Saudi Arabia	73	0	0.00
Southwestern Saudi Arabia	27	7	0.1429
South Sinai, Egypt	43	0	0.00
North of Iran	3	33	0.6666
Turkey	6	100	0.3334
Syria	2	0	0.00
Yemen	3	0	0.00
Greece	2	50	0.00
Jordan	1	0	0.00

Table 4.4: Table indicating SNPs (shown in red) that generate the different haplotypes of *Alkanna orientalis* based on chloroplast DNA (trnH-psbA region). The two main haplogroups subsequently identified in the network analysis are shown in green and yellow respectively.

H1	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C A T T T T T T T T T T T A A A G G G C G A G A G G G T	37
H2	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C T T T T T T T T A T T T T A A A G G G C G A G A G G G T	32
H3	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C T T T T T T T T T T T T T A A A G G G C G A G A G G G T	23
H4	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G T A T T T A G G T G T T T C T C C C T T T T T T T T T T T T T A A A G G G C G A G A G G G T	46
H5	A G G G A C G G A C G A A A C A A C C T A A A T G C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A C T C T G A A G A A G T A T A T A A C C	1
H6	A G G G A C G T A A G A A A C A A C C T A A A T A C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A C T C T G A A G A A G T A T A T A A C C	14
H7	A G G G A C G G A C G A A A C A A C C T A A A T A C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A C T C T G A A G A A G T A T A T A A C C	1
H8	A G G G A C G T A A G A A A C A A C C T A A A T C C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A A T T C T G A A G A A G T A T A T A A C C	20
H9	A G G G A C G T A A G A A A C A A C C T A A A T C C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A A T T C T G A A G A A G T A T A T A A C C	2
H10	A G G G A C G T A C G A A A C A A C C T A A A T C C A T C A A T C C C C T C T G T A A A A A G A A A C A A A A A A T T C T G A A G A A G T A T A T A A C C	1
H11	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C T T T A T T T T T T T T A A A G G G C G A G A G G G T	1
H12	T T A C A T C T T A T T T T A G C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C T T T T T T T T T T T T T A A A G G G C G A G A G G G T	1
H13	T T A C A T C T T A T T T T A A C A G A G G G G A T T G A T G G A T T T A G G T G T T T C T C C C T T T T T T T T T T T T T A A A A G G G C G A G A G G G T	1

Table 4.5: Summary statistics for chloroplast DNA haplotypes (trnH-psbA region). Saudi Arabian populations are shown individually and with populations from the two regions (northwestern and southwestern) grouped together.

Population	N		Private haplotype %		Gene diversity	
Northwestern SA – Al-Loz	61		0		0.722	
Northwestern SA – Ash-Shyati	8	76	0	0	0.607	0.734
Northwestern SA – Al-Ward	7		0		0	
Southwestern SA – Mana'a	15		13		0.371	
Southwestern SA – Nahran	11	39	0	100	0.345	0.656
Southwestern SA – Al-Sawda	13		8		0.294	
South Sinai, Egypt	39		0		0.658	
Northern Iran	3		0		0.666	
Turkey	15		13		0.257	
Syria	1		0		0	
Yemen	3		0		0	
Greece	3		33		1	
Jordan	1		0		0	

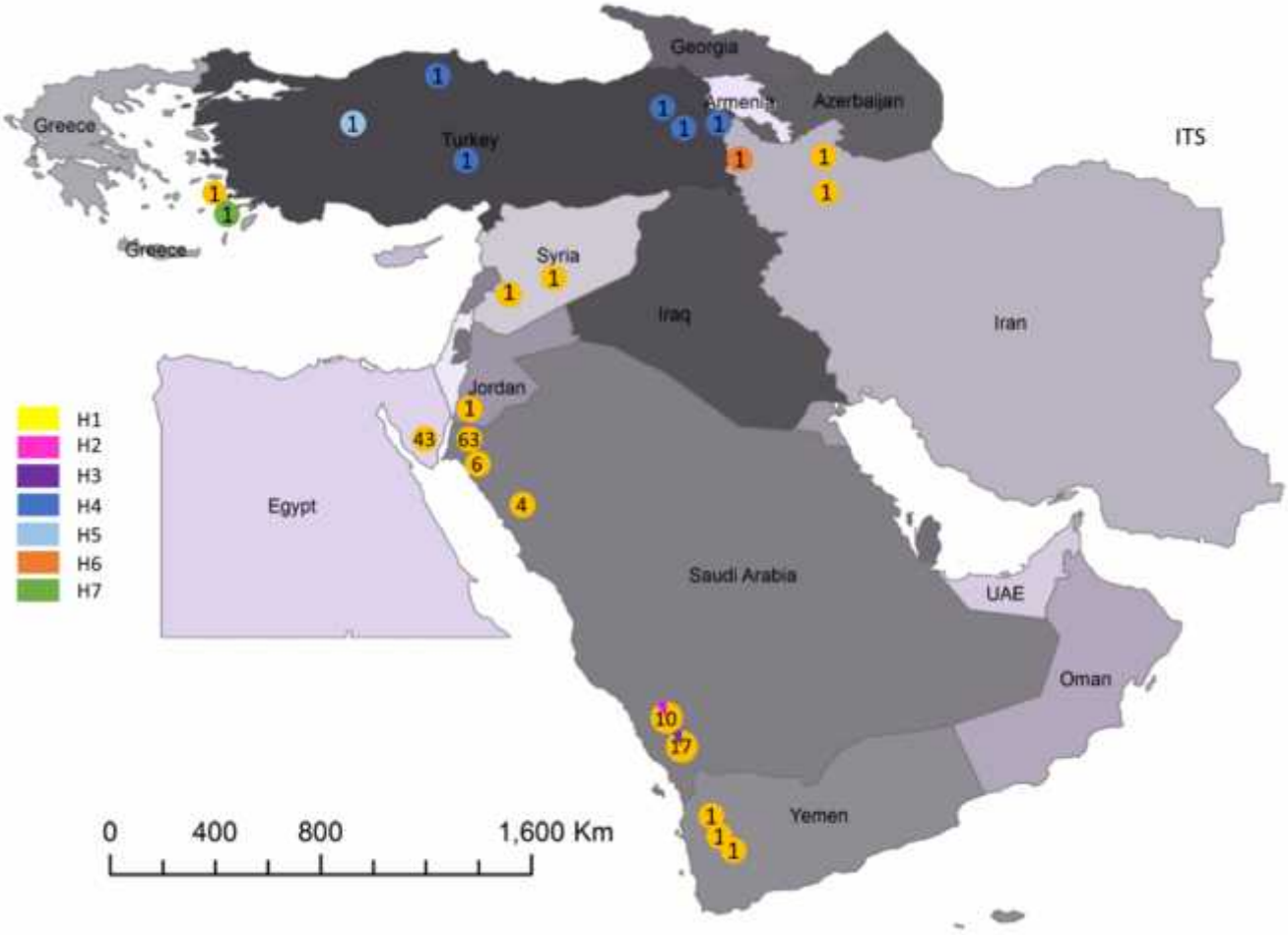


Figure 4.1: Map showing eight countries where *Alkanna orientalis* were sampled or obtained for this study. The circles represent the population genetic structure based on nuclear ribosomal DNA (ITS region) across the eight countries. The numbers inside the circles indicate the sample size for each population.

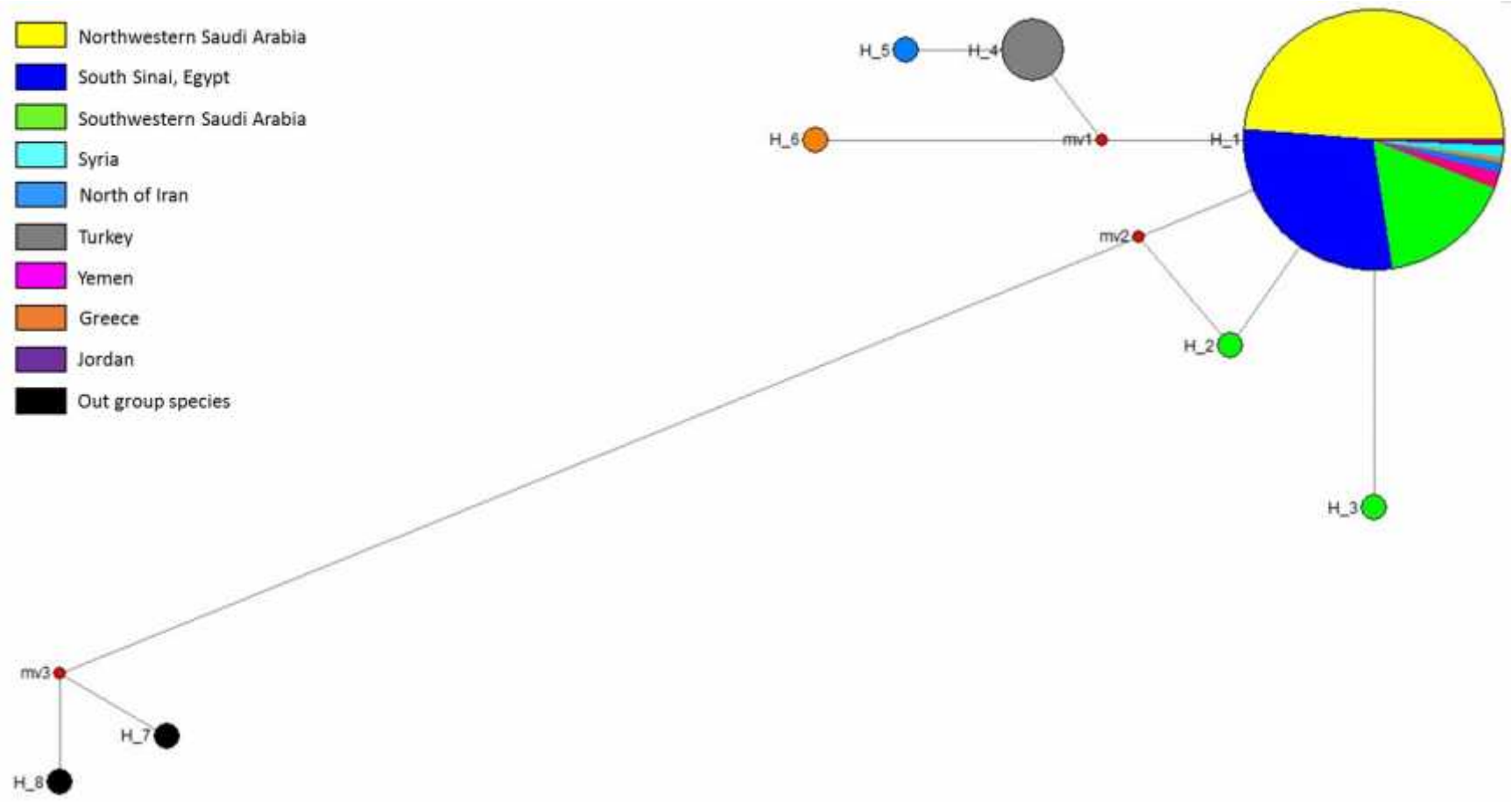


Figure 4.2: Nuclear ribosomal DNA (ITS region) haplogroups in *Alkanna orientalis*. Small red circles represent nodes, which correspond with inferred missing haplotypes or homoplasies. Branch lengths are proportional to the number of mutations. The haplotypes are coloured regarding to their nine geographical groups. The outgroup species are *Alkanna tinctoria* and *Alkanna sieberi*. Circle size is proportion haplotype frequency.

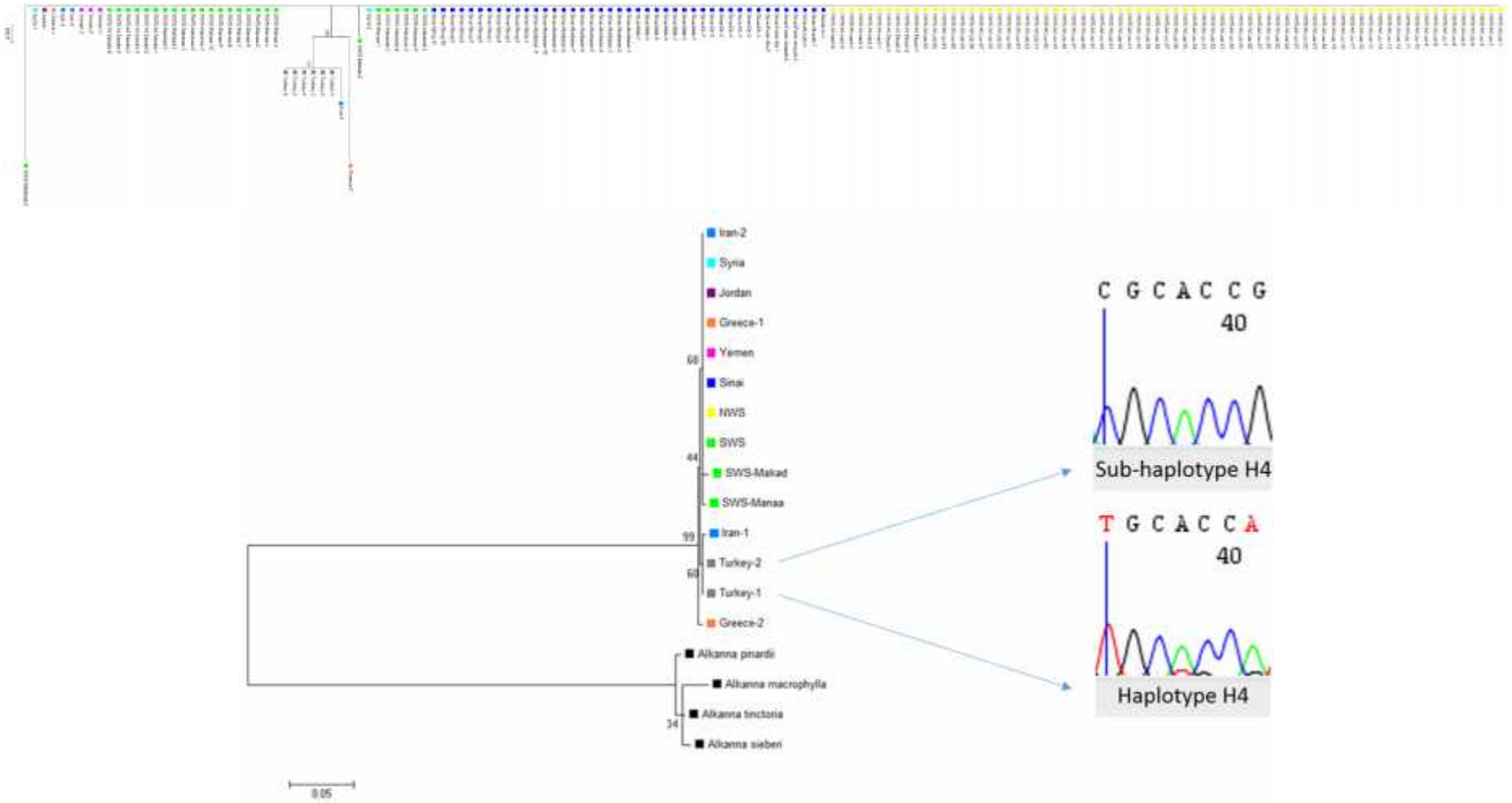


Figure 4.3: Genetic relationship between *Alkanna orientalis* based on nuclear ribosomal DNA in ITS region over Middle East range and eastern Mediterranean. The related outgroup species were selected from GenBank based on BLAST searching for closest sequence matches.

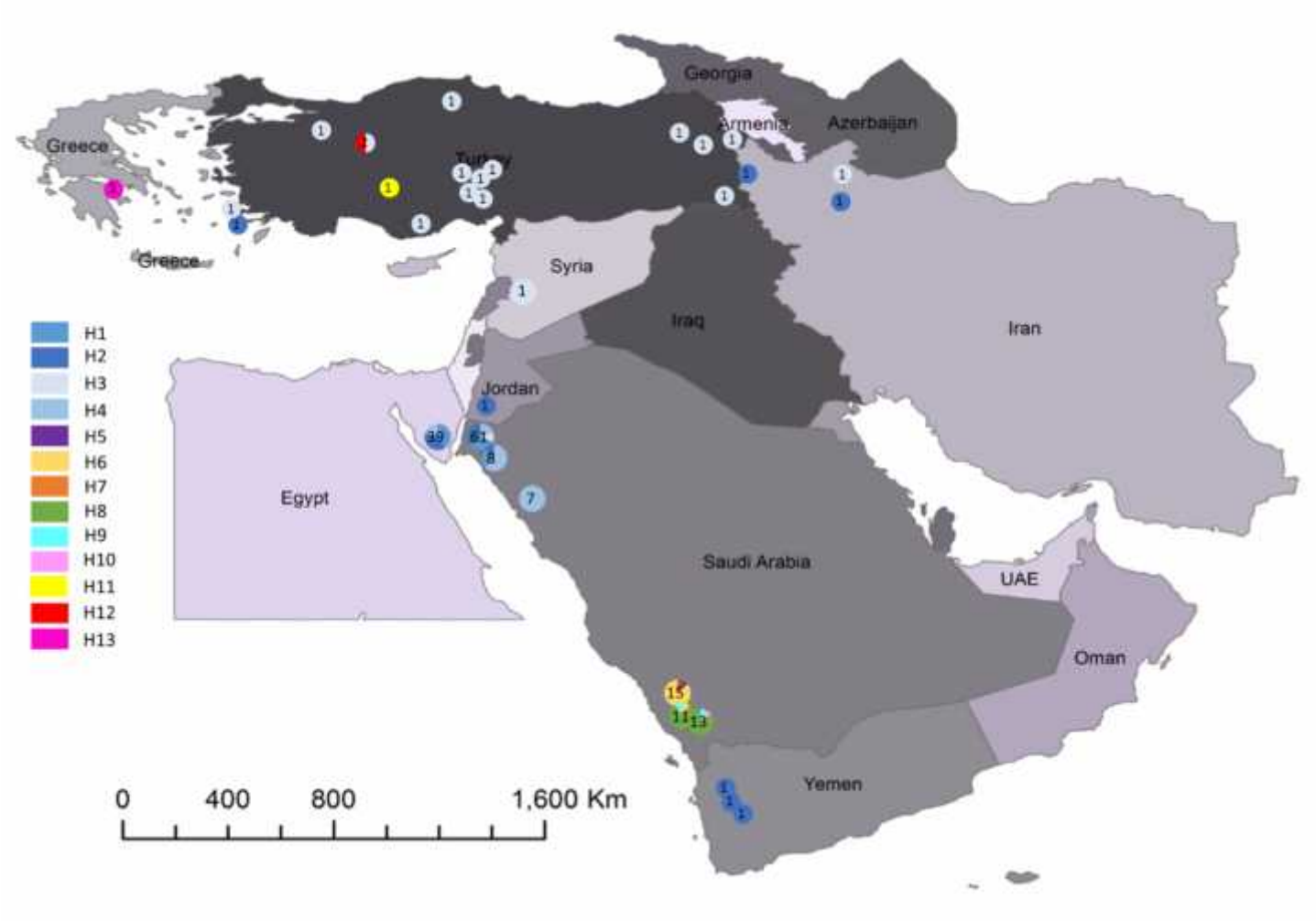


Figure 4.4: Map showing eight countries where *Alkanna orientalis* were sampled or obtained for this study. Each circle represents the origins of samples with the number of individuals has been analysed based on chloroplast DNA (trnH-psbA region).

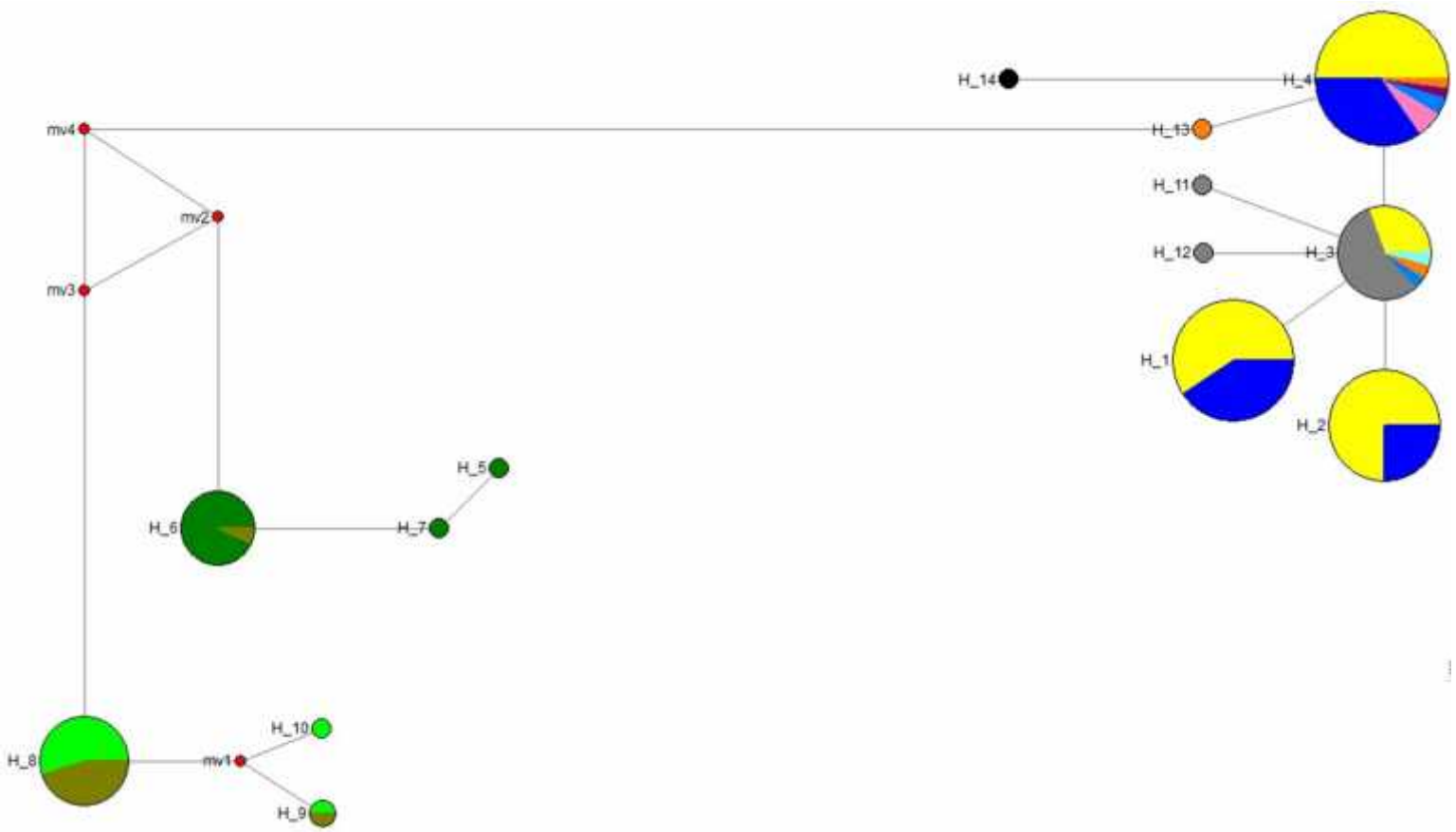


Figure 4.5: Chloroplast DNA (trnH-psbA region) haplogroups in the Middle East range identified from 180 specimen *Alkanna orientalis* and an outgroup species (*Alkanna tinctorial*; black circle). Small red circles represent nodes, which correspond with missing haplotypes or homoplasies. Branch lengths are proportional to the number of mutations. The haplotypes are coloured regarding to their thirteen geographical groups. Circle size is proportional to haplotype frequency.

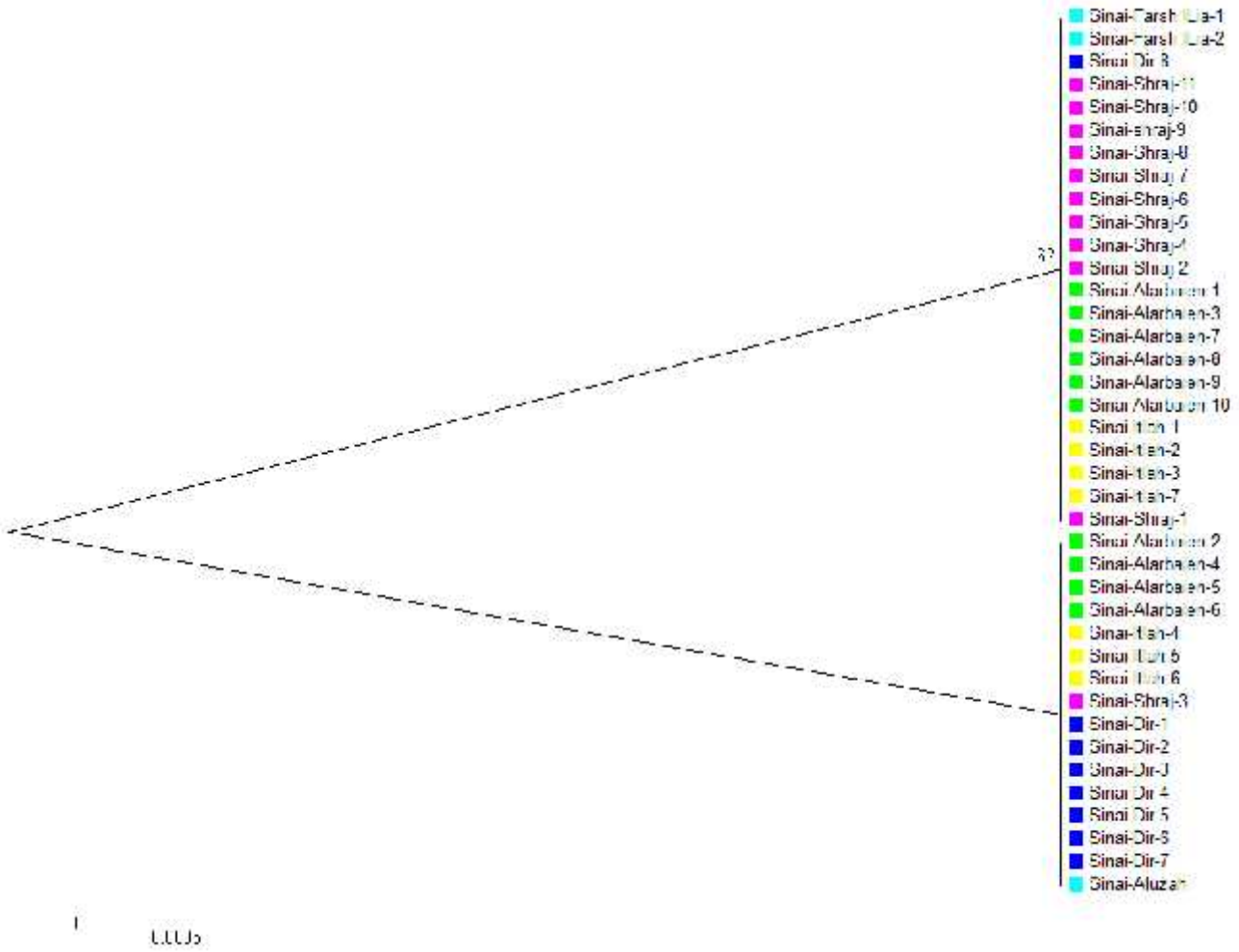


Figure 4.6: Neighbour-joining dendrogram of the individual *Alkanna orientalis* based on chloroplast DNA in trnH-psbA region. Individuals are from different wadis at St Katherine, south Sinai. Each colour represents a different wadi.

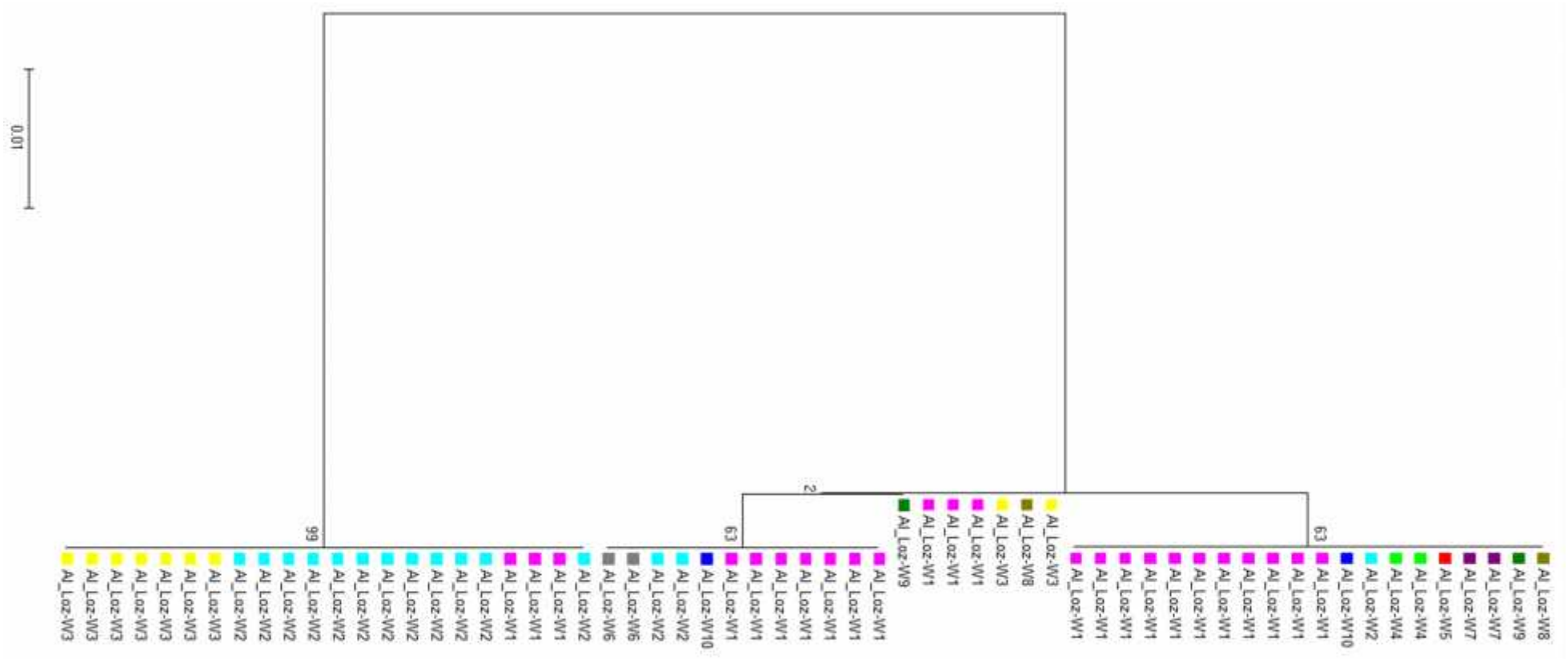


Figure 4.7: Neighbour-joining dendrogram of the individual *Alkanna orientalis* based on chloroplast DNA in trnH-psbA region. Individuals are from different wadis at Jabal Al-Loz, northwestern Saudi Arabia. Each colour represents a different wadi.

4.5 Discussion

This is the first study of *Alkanna orientalis* using both paternally and maternally inherited genetic markers, with the aim being to understand the population genetic structure of a wild plant that is widespread in the eastern Mediterranean and Middle East regions. From the point of view of the nuclear genetic markers, the Turkish population stands out as being different from the rest, and with a higher degree of diversity compared to other populations because all individuals sampled were unique (Fig. 4.1). A further noticeable finding is the separation of *A. orientalis* into two lineages according to longitude across the eastern Mediterranean region. Such a phylogeographic separation pattern seems to be common in a direction from the west to the east Mediterranean (Kadereit *et al.*, 2008; Casimiro-Soriguer *et al.*, 2010; Feliner, 2014; Santis *et al.*, 2016) although other studies interpret genetic differentiation as having an east to west pattern (Lumaret *et al.*, 2002; Escudero *et al.*, 2010; Migliore *et al.*, 2012). The pattern of separation in *A. orientalis* may be the result of the natural topography of Turkey and the surrounding region, which is surrounded by high elevated mountain barriers and bordered by the Black Sea and the Mediterranean. Such barriers can effect long-term isolation of populations from other parts of the range and thus facilitate their differentiation from other regions. In contrast, regions of the Middle East have possibly been exposed to demographic changes, such as range contraction and expansion, which have acted to diminish genetic diversity.

From the point of view of the cpDNA molecular markers, genetic variants were found to be shared among most countries except the populations from southwest Saudi Arabia, where the genetic types were unique. Thus the most interesting aspect here is the phylogeographic pattern where the eastern Mediterranean shares a common

haplotype with the north and south Middle East, but is distinct from the haplotypes found exclusively in the populations from southwest Saudi Arabia. One possible explanation for the latter diversity is that there has been introgression through hybridization, which has caused the diversity in southwestern Saudi Arabia populations.

Both nuclear and chloroplast markers studied here appear to indicate that the *Alkanna orientalis* clades have originated in the far-eastern Mediterranean region, with derived genetic types occurring in the western Mediterranean and Middle East regions. This pattern agrees with previous studies that have proposed the eastern Mediterranean to be a glacial refugium area (Hewitt, 1999; Méd *et al.*, 2009). In addition, a gradient of reduced diversity from the Mediterranean towards the Middle East region appears consistent with southward colonisation out from the Mediterranean refugial areas.

Using a maternally inherited marker that can be dispersed through the maternal but not the paternal line (i.e. through seeds but not pollen) can assist in evaluating the relative contributions of gene flow through seeds and pollen (Ennos, 1994). Here in our study, I found that the biparentally inherited marker (nrDNA, ITS region) is less variable than the maternally inherited marker (cpDNA, trnH-psbA region) among and within *A. orientalis* plant populations. Clustering of individuals from all populations based upon cpDNA sequences, as is found here, illustrates that whilst there may be long-distance gene flow within and between populations mediated by movement of pollen, the same may not be true for maternally inherited genes, which can only be transported by seeds. The main pollinator of the plant in Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1996) and at Jabal Al-Loz in Saudi Arabia (second chapter) is *Anthophora pauperata*. Thus at a small spatial scale, I can interpret the low genetic differentiation of nuclear genes among

populations to be accounted for by pollinator-mediated gene flow. I have shown (previous chapter) that *Anthophora pauperata* has the ability to travel for long distances carrying pollen between plants in different wadis. However, currently I have no evidence that *Anthophora pauperata* can travel even longer distances across between populations in different regions, as other pollinators are known to do; e.g., some wasps can disperse pollen over long distances by being blown by wind (Liu *et al.*, 2015).

The small size of *Alkanna* seeds means that potentially they can easily be moved via water during floods, but there is no evidence of animal-mediated seed dispersal. In the case of movement by water, maternal gene flow will follow the direction of water flow, while gene movement of pollen via pollinators will be in whichever direction they prefer. In the case of the cpDNA, the expectation is that gene flow can occur only through seeds. Accordingly, genetic diversity was greater within and between populations, and this might be due to the lower effectiveness of gene flow through seeds relative to pollen. Furthermore, the more private haplotypes in southwest Saudi Arabia might demonstrate a higher ability in the region to diverge and spread. The mountainous nature might also explain the divergence between populations at local scales (e.g. on either side of a mountain) and at larger scale (e.g. across mountain ranges). Historical introgression of chloroplast genes from a closely-related species could also explain the localised occurrence of diverged genetic variants.

There are many and varied arguments from genetics and ecological approaches about conservation prioritisation at species and population levels (Schemske *et al*., 1994; Petit *et al*., 1998; Arponen, 2012; Winter *et al*., 2013). Recent arguments have been made for conservation strategies to be prioritized in areas with high numbers of genetic variants (allelic richness) and areas with genetic uniqueness (genetic divergence)

because of their contribution to the entire diversity (Petit *et al*., 1998; Médail *et al*., 1999; Arponen, 2012). However, arguments have also been made for populations under anthropogenic threats to receive the high prioritization because the timescale of occurrence and conservation actions is much shorter than any evolutionary process.

Broadly speaking, human beings constitute the main driver of threats to biodiversity (Pimm *et al.*, 2014). In our study, it could be argued that the eastern Mediterranean should have the highest conservation priority, as a refugial area population and because of the extent of human impact. This is followed by southwest Saudi Arabia, which has a high number of genetic variants that are strongly diverged from other populations, and is an area suffering under the impact of human population growth. Pleistocene glacial features have been described in the highlands of Yemen (El-Nakhal, 1993) and recently in the Madiyan region of Saudi Arabia (Laboun, 2012). Such indications are important in terms of conservation since these regions might be refugial areas. We do not know if Pleistocene glaciation also occurred in the Sinai region, but the topography and outcrops of Sinai region are very similar to those of the Madiyan region of Saudi Arabia.

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Chapter 5: A population and phylogenetic analysis of an Anthophorid bee: *Anthophora pauperata* (Boraginaceae) in Saudi Arabia and Sinai

5.1 Abstract

The worldwide decline of bees is a concern for many conservationists and other ecologists, but the situation for solitary bees, many of them dietary specialists, may be of particular significance, especially if there is evidence of loss of intraspecific diversity. *Anthophora pauperata* is an early spring species that concentrates on the flowers of *Alkanna orientalis*. It is endemic to a small area of the St Katherine Protectorate (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Here I characterise genetic variation within and amongst populations of this species over small and large spatial scales by sequencing a protein coding region of mitochondrial DNA, and by genotyping ten hyper-variable microsatellite loci. I find evidence of substantial genetic differentiation between populations over large geographical scales among populations, but evidence of significant gene flow within populations over smaller geographical scales, such as between wadis. The conservation consequences of these patterns are discussed.

Key words: solitary bees, *Anthophora pauperata*, genetic variation, phylogeography, conservation

5.2 Introduction

The world-wide loss of biodiversity has led to considerable interest in understanding how to minimize resulting ecosystem dysfunctioning, for example through loss of ecosystem services such as pollination (Loreau *et al.*, 2001; Hooper *et al.*, 2005). Insect pollinators are responsible for at least 35% of world food productio (Klein *et al.*, 2007) but the misuse of the landscape by human activities, which leads to habitat fragmentation, habitat loss, and the use of agrochemicals, has had negative effects on pollinator diversity (Steffan-Dewenter *et al.*, 2002; Ricke *et al.*, 2008; Gri *et al.*, 2009; Pot *et al.*, 2010). There are many studies illustrating the degree to which pollinators are declining, in particular the bees (Hymenoptera: Apoidea), where global reductions have led to serious economic effects in agriculture (Biesmeijer *et al.*, 2006; Brading *et al.*, 2009; Grix *et al.*, 2009; Pot *et al.*, 2010; Pauw *et al.*, 2011).

Within the context of biodiversity loss, the importance of intraspecific genetic diversity for long-term survival of threatened species remains an on-going topic of discussion (eg. Frankel, 1970). One mechanism through which genetic diversity may be related to population decline as if there is a decline in fitness due to an absence of heterozygosity (Hansson *et al.*, 2002). Specialist species may be more vulnerable because they are involved in mutually dependent relationships in comparison with generalist species (Packer *et al.*, 2005; Clavel *et al.*, 2011).

A variety of molecular genetic marker types can be used to assess the level of intraspecific genetic diversity. However, selecting an appropriate marker is contingent on the nature of studies and the accessibility of genetic tools for the particular species to be studied. Short DNA sequences of the mitochondrial gene (mtDNA) cytochrome c

oxidase subunit 1 (CO1) have been shown to be efficient molecular markers for characterising biological diversity below the species level (Rubinoff, 2006; Hubert *et al.*, 2008). Microsatellites (repetitive sections of DNA) have also been used widely as an informative tool to study genetic diversity and population structure in many types of animal (reviewed by Allendorf *et al.*, 2008 for wild animals; and for domestic animals by Cymbron *et al.*, 2005; Canon *et al.*, 2006; Sancristobal *et al.*, 2006; Peter *et al.*, 2007; Khanshour *et al.*, 2013) and in social (Estoup *et al.*, 1995; Herrmann *et al.*, 2007) and solitary (Zayed *et al.*, 2007; Davis *et al.*, 2010; López-Uribe *et al.*, 2013) bees in particular

Previous genetic studies of bees were based on enzyme polymophisms (Snyder, 1974; Pamilo *et al.*, 1978; Carman *et al.*, 1996) and RAPD markers (Lu *et al.*, 1996; Tavares *et al.*, 2007), which were used to diffe genetically amongst geographically isolated populations (Lu *et al.*, 1996; Jain *et al.*, 2010). In the last decades, however, many mtDNA sequences (Soucy *et al.*, 2002; Solorzano *et al.*, 2009; Ferreira *et al.*, 2013) and microsatellite loci (Lacey, 2001; Francini *et al.*, 2010; Francisco *et al.*, 2011; Zimmermann *et al.*, 2011; Cerna *et al.*, 2012) have been used. Microsatellites are ofte the most suitable marker to study bi-parentally inherited genetic diversity within and among populations, whereas mtDNA, which is maternally inherited (Arif *et al.,* 2009), may be more appropraite for determining phylogenetic relationships among closely related species.

A previous study using RAPDs suggested that *Anthophora pauperata* bees from adjacent wadis in South Sinai were genetically different from each other, even though the wadis are only a few kilometres apart (Mahmoud *et al.*, 2008), but there were no other data available on the genetic relationships among widely separated populations. Thus the ultimate goal for this study is to understand the genetic relationships and hence

the history of *A. pauperata* populations among and within the two known areas that they occupy, i.e. those of the St Katherine Protectorate in South Sinai and a new population discovered in Jabal Al-Loz, in northwestern Saudi Arabia. To establish the level of genetic diversity, I sequenced the maternally inherited mitochondrial cytochrome oxidase subunit I & II and tRNA region (Hall *et al.*, 1991), and ten hyper-variable, biparentally inherited microsatellite loci that have been used as genetic markers in related species (Cerna *et al.*, 2012).

5.3 Material and methods

5.3.1 Study area

Fieldwork took place throughout June 2012 and 2015 in St. Katherine, Sinai, Egypt and during March 2014 at the following locations in Saudi Arabia: Jabal El-Loz and Jabal Ash-Shyati (Madiyan mountain range, Tabuk region) in the northern part of the western highlands of Saudi Arabia (N 28º 43.452' E 35º 19.417'); and Jabal Mana, Jabal Nahran and Jabal Al-Sawda (Al-Sarawat mountain range, Asir region) in the southern part of the western highlands of Saudi Arabia (N 18º 58.09' E 42º 11.02'/ N 18º 10.13' E 42º 27.02'/N 18º 20.21' E 42º 20.37'). These regions of Saudi Arabia and Sinai comprise high mountains with narrow and wide, open wadis. I started the field surveys from 1200 m above sea level on each mountain, climbing until I reached the top. In each wadi, I surveyed a patch of approximately 100 x 100 m, and then continued along the surveying patches every 200-300 m.

5.3.2 Study system

The study focussed on *Anthophora pauperata* (Walker 1871) (Apoidea: Anthophoridae), a species previously thought to be endemic to the South Sinai mountains (Mahmoud *et al.*, 2008), but recently also discovered in Jabal Al-Loz (this study). *A. pauperata* is the main visitor and the only pollinator of *Alkanna orientalis* (L.) Boiss., 1844 (Boraginaceae) in South Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1996) and at Jabal Al-Loz in Saudi Arabia (see chapter 2). *A. pauperata* appears at the time of the main flowering of *Alkanna orientalis*. It first appears during early March at Jabal Al-Loz (chapter 2) and during late March at St Katherine (Zalat, 1984; Willmer *et al.*, 1994). Males are territorial with a unimodal diurnal activity pattern in 'guarding' or patrolling a number of blooming *Alkanna* plants, while females show bimodal diurnal foraging activity in the early morning and late afternoon, probably digging and maintaining their nests in between these two periods. Matings were observed during the short periods when males and females coincided in the early morning and late afternoon (Chapter 2; Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Gilbert *et al.*, 1999).

5.3.3 Sampling

No bees where found on *Alkanna* plants at Jabal Al- Shyati, and they were very infrequent at Jabal Mana'a, where only one bee could be found for sampling: sequence data indicated that this was not *A. pauperata* [possibly an undescribed species]. A total of 40 bee specimens of *A. pauperata* were sampled from different wadis in south Sinai (Si population): Arbaien, Shraj, El-Dir, Itlah, Raha and Abu Seila (Fig. 5.1). A total of 38 *A. pauperata* specimens were sampled from different wadis at Jabal Al-Loz (Ns population). Those specimens were sampled from sites W1A, W1B, W2A, W2B, W3A, W3B, and W4A (Fig. 5.2). None of the 60 bee specimens sampled from Jabal Nahran and Jabal Al-Sawda

were identified as being *A. pauperata* on the basis of subsequent morphological identification.

5.3.4 DNA extraction and purification

One leg of each bee was stored in absolute alcohol prior to DNA extraction. The leg tissue was ground in a 1.5 µl Eppendorf tı ising a disposable plastic pestle, followed by DNA extraction using a Sigma Aldrich DNeasy kit, which uses a selective column to bind DNA preferentially prior to elution. DNA samples were stored at −20°C and diluted 1/100 (to dilute potential inhibitors) f first attempts to use the solution as a template for PCR were unsuccessful.

5.3.5 Genetic markers

5.3.5.1 Mitochondrial DNA

To examine the genetic diversity and structure among *Anthophora* populations, an approximately 800-bp encoding sequence of mitochondrial DNA (cytochrome c oxidase subunit I and II) was amplified using published primers (Hall *et al.*, 1991). PCR was carried out in a total volume of 30 µl for all specimens utilising a Reddymix™ master mix for PCR reaction (supplied by Fisher). This total volume consists of 3 µl DNA used as a template for the PCR and a final $MgCl_2$ concentration of 2.5 mM. Annealing temperatures between 50°C and 60°C were tested for each primer in order to establish the most suitable reaction conditions. The PCR cycles of mtDNA involved an initial denaturation step at 95°C for 2 min, followed by 40 cycles, each comprising of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and 30 sec, extension at 72°C for 2 min and followed by a final extension of 72°C for 7 min and 30 sec.

PCR products were separated on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet light to indicate if amplification had been successful. Successfully amplified PCR products were sent to Macrogen Company for sequencing. CodonCode Aligner v 6.0.2 were used to identify variation amongst sequences. BLAS searching (Camacho, 2013) was used to identify sequences from other closely-related species that have already been deposited on Genbank (Altschul *et al.*, 1997).

Haplotypes were constructed from DNA sequences using the software DnaSP v.5 (Librado *et al.*, 2009). Gene diversities were calculated for each population using the standard formula:

$$(n/[n-1])(1-\sum_{i}^{k} P_i^2)$$

where n constitutes sample size, k the number of different haplotypes and P_i the frequency of haplotype i. Phylogenetic Network software (Bandelt *et al*., 1999) was used to generate evolutionary trees and networks. Phylogenetic trees were constructed using the methods implemented in the software MEGA (Tamura *et al.*, 2007).

5.3.5.2 Microsatellites

Ten microsatellite loci (Table 5.1) were selected out of 37 that have previously been used for *Anthophora plumipes* and shown to be successful in other related species (Cerna *et al.*, 2012). A total of 78 *Anthophora* samples representing two populations of south Sinai (Si) and Jabal Al-Loz (Ns) were analysed. The sample size and place of origin of each genotyped population are listed in Table 5.2.

To lay out and adjust multiplex PCRs, the nominated ten microsatellite loci were assessed utilising the Multiplex Manager program V. 1 (Holleley *et al.*, 2009). Based on the given data of primer condition, Multiplex Manager generates novel multiplex

reactions by reducing the entire number of reactions, exploiting the spacing between markers in a single reaction and reducing the disparity in annealing temperature for each reaction. The forward primers were fluorescently labelled (using 6-FAM, NED or PET respectively). Two PCR multiplexes were effectively generated with 10 loci (Table 5.3).

The polymerase chain reaction (PCR) for the ten loci was carried out in a total volume of 10 µl for all specimens utilising a Reddymix™ master mix for PCR reaction (supplied by Fisher). This total volume consists of 1 µl DNA used as a template for the PCR and a final $MgCl_2$ concentration of 2.5 mM. Annealing temperatures between 50° and 60°C were tested for each multiplex in order to establish the most suitable reactic conditions. The PCR cycles for all loci involved the initial denaturation step at 95°C for 15 min, followed by 30 cycles, each comprising of denaturation at 94°C for 3 annealing at 60°C for 90 sec, extension at 72°C for 30 sec and followed by a final extension of 60°C for 30 min.

PCR products were separated on 1.5% agarose gel containing ethidium bromide and visualized under ultra violet light to indicate if amplification had been successful and to prepare an appropriate dilution for each multiplex. Successful PCR products were sent to the DNA sequencing Lab at the Queen's Medical Centre (QMC), Nottingham for genotyping on an ABI capillary sequencer, using a ROX™ size standard.

5.3.6 Statistical analysis

5.3.6.1 Molecular markers

For each of the ten microsatellite loci, I calculated the entire number of alleles (N_A), the observed (H_O) and expected (H_E) heterozygosity and the polymorphism information content (PIC, which assesses the genetic diversity within species for each locus and

hence sheds light on the evolutionary process that the locus might experience through time), using the approaches implemented in the software CERVUS V. 3.0 (Kalinowski *et al.*, 2007). The software also tested the possibility that the allele frequencies deviate from Hardy–Weinberg equilibrium (HWE). Departure from HWE is an indicator that the assumptions of random mating within a stable population are not valid, or it could indicate the presence of null alleles. The Brookfield method (Brookfield, 1996) was used to test for the presence of null alleles, an allele with a genetic mutation in a primer site that prevents amplification.

5.3.6.2 Population structure

To evaluate the degree to which populations are subdivided, analysis was made using the approaches implemented in the program STRUCTURE, which assigns individuals into particular genetic clusters and carries out an admixture analysis utilising a Bayesian clustering method (STRUCTURE V. 2.3.3, Pritchard *et al.*, 2000; Falush *et al.*, 2007). This approach identifies populations that are genetically distinct and defines migrants and admixed individuals. In this analysis, different *K* values were tested (from *K* = 2 to *K* = 7), where *K* is the number of putative genetic clusters, by applying a burn-in of 10,000 followed by 50,000 Markov Chain Monte Carlo (MCMC) iterations. Ten repetitions were executed for each *K* with the assorted ancestry (admixture model) analysed with the CLUMPP program to align the multiple replicates and to strengthen the interpretation of the clustering result (Jakobsson *et al.*, 2007). Yields from STRUCTURE were evaluated in STRUCTURE HARVESTER (Earl, 2012); the ideal number of clusters was specified according to the method of Evanno *et al.* (2005) and Pritchard *et al.* (2000)'s recommendation. The output graph of the replicated result was produced using the program DISTRICT v. 1.1 (Rosenberg, 2004).

To test for the existence of recent reductions in population size, I applied the program BOTTLENECK V. 1.2.02 (Piry *et al.*, 1999), which tests for an overall allele excess relative to the number of heterozygotes. I evaluated the data under the two models of microsatellite mutation: the stepwise mutation model (SMM) and the infinite alleles model (IAM). Under SMM, mutations modify an existing allelic state with equal probability of one step forward or backward. The same allelic identity can therefore arise more than once, if there is an increase in size followed by a decrease in size back to the original state. Under the IAM, each mutation is considered to result in a new allele and thus alleles of the same size are assumed to identical by descent. Use of both models is recommended because the reality is that most loci probably evolve in a way that is intermediate between the two models (Di Rienzo *et al.*, 1994). Genepop software version 4.5 (Rousset, 2008) was used to calculate the allele frequencies in both populations.

5.4 Results

5.4.1 mtDNA

Three regions of mtDNA were assayed in 78 individuals of *Anthophora pauperata*. The total numbers of individuals that were successfully sequenced varied based on the amplified region of mtDNA: 60 for the 204-bp encoding region of COI (68 amino acids); 33 for the 365-bp encoding region of COII (121 amino acids); and also 33 at the tRNA non-coding region in-between COI and COII of about 209-bp.

Polymorphic sites (SNPs) were identified in all regions. Four SNPs were identified in the COI region (see Table 5.4), one of which causes an amino acid substitution (from

phenylalanine to leucine). Five SNPs were identified in the COII region (see Table 5.4), all of which are silent substitutions. The most polymorphic region was tRNA with 11 SNPs (see Table 5.4).

The total number of haplotypes were three (COI), six (COII) and two (tRNA): their geographic distributions are shown in Figures 5.3, 5.4, and 5.5 respectively. Sev haplotypes were identified when I combined all three regions together (Fig. 5.6). Interestingly, all haplotypes were private, i.e. restricted to a single country within the region studied, but were shared across sites within a country.

Measures of genetic variation and the number of private haplotypes observed within each population for the COI, COII, and tRNA regions are shown in Table 5.5. The genetic differentiation among populations for COI, COII, and tRNA regions was estimated as F_{st} = 0.99, F_{st} = 0.88, and F_{st} = 1.00 respectively (significance estimated by permutation, 10,000 replicates $P << 0.0001$).

5.4.2 Microsatellites

From the ten selected microsatellites I identified a total of 38 alleles among the genotyped individuals (Table 5.6), whose frequencies shown in Figure 5.7. The majority of the loci were informative, showing a substantial degree of polymorphism. The mean number of alleles per locus is 3.8 (range 2-7). The mean polymorphism informati content (PIC) value was 0.357 over all loci. The highest and lowest expected heterozygosities were 0.799 (anth40a) and 0.028 (anth35), respectively. Mul analyses for all markers considering the two populations indicate that four out of ten are in a Hardy–Weinberg equilibrium. The estimated frequency of null alleles, which are

expected to cause apparent deviations from Hardy-Weinberg, ranges from 0.405 to 0.988.

Six microsatellite loci (anth75, anth74, anth72, anth64, anth43 and anth40a) were polymorphic within and between the two populations (Ns & Si). Two (anth7 anth35) were polymorphic within the Ns population and monomorphic within th population. Two (anth30 & anth24) were polymorphic within the Si population monomorphic within the Ns population. Most of the loci showed significant genetic differentiation among the two populations and within the Si population (Table 5.7); while two loci showed significant differentiation within the Ns population.

To define the probable number of genetic clusters in the dataset, I implemented STRUCTURE analysis. Initially, I scrutinised the highest level of population structure for the two *Anthophora* populations. STRUCTURE HARVESTER analysis showed that the ideal number of genetic clusters is two (Fig. 5.8). This level of structuring represents the Ns bees as one population, but the Si bees as two populations (Fig. 5.9). Modifying the value of *K* to be three permits differentiation between the two main populations, with some individuals in the Ns population representing individuals from the Si population (Fig.5.9). No additional genetic structure was detected above *K* = 3.

The mean number of individuals examined per locus was 36 (Ns) and 40 (Si), with eight polymorphic loci in each. This is slightly larger than the minimum number (20-30 individuals, 5 polymorphic loci) needed for the reliability of the sign test (Luikart *et al.*, 1998). Significant heterozygote excess was calculated in both populations under both the Infinite Allele Mutation and Stepwise Mutation models using three different tests (Table 5.8). Sinai population showed evidence of a recent bottleneck under both IAM

and SM models in all three tests, while the SA showed evidence of a recent bottleneck only under the SMM model in all three tests (Table 5.8).

Table 5.1: The selected microsatellite loci for *Anthophora pauperata* from related species.

NO	Locus	Source species of locus	Reference
1	anth24	Anthophoridae	(Cerna *et al.*, 2012)
2	anth30		
3	anth35		
4	anth40a		
5	anth43		
6	anth64		
7	anth72		
8	anth73		
9	anth74		
10	anth75		

Table 5.2: List of *Anthophora* populations genotyped.

Populations	Wadi	Sample size
South Sinai, Egypt	wadi Arbaien	10
	wadi Shraj	10
	wadi El-Dir	7
	wadi Itlah	5
	wadi Raha	7
	wadi Abu Seila	1
Jabal Al-Loz, north west Saudi Arabia	wadi 1A	6
	wadi 1B	6
	wadi 2A	8
	wadi 2B	8
	wadi 3A	4
	wadi 3B	4
	wadi 4A	2

Table 5.3: Primer sequences of the 10 microsatellites and fluorescent tag used.

Locus	Repeat $()_n$	Multiplex	Primer sequence 5' to 3'	Dye	Size range
anth24_F	(TC)12		GATTTCGCGACTAGACCAGC	6-FAM	112-122
anth24_R			GTTTGACCTTCGCTACCTTCCGTA		
anth30_F	(CA)8		CAGGCAGGACAGAAACATCA	PET	105-113
anth30_R			GTTTGTGGTCCGTCGAGTGGTATG		
anth35_F	(TTG)10	2	TTGGAATCCATGTTGCACAG	PET	176-185
anth35_R			GTTTACATGCGTGGATGACACAGT		
anth40a_F	(CA)13		CAAGCCCATGTTTTCCTCTC	NED	219-219
anth40a_R			GTTTACGAAGCGATACCACGTCTC		
anth43_F	(TCT)19		GAACGCAACGAATCGAAGAC	6-FAM	187-193
anth43_R			GTTTGGTGCATCGATAGCCTGTA		
anth64_F	(GA)15		GATCATAAATCAACGGGTACGA	NED	143-165
anth64_R			GTTTCGCTTAGTTACTCAACAAAGACAAC		
anth72_F	(TTC)11		ATTGGACGCTTTAATGTCGG	NED	223-232
anth72_R			GTTTAGAACTAACGAGCCAGCCAG		
anth73_F	(GAG)8	1	GGTTATCGAAACGTCGAGGA	PET	218-224
anth73_R			GTTTGCACAGCCTGTAATTACGCA		
anth74_F	(TCG)14		CATCTTGGGTTCGTCCAGTT	NED	86-102
anth74_R			GTTTGTCGCCGTTACATCCGAG		
anth75_F	(CTT)10		CACCGATTTTAAACCGCTAA	6-FAM	80-92
anth75_R			GTTTCCGGTATCTGAACGTCAAC		

Table 5.4: SNPs that generate the different haplotypes of *Anthophora pauperata* based on mtDNA (cytochrome c oxidase subunit I & II and tRNA regions). The highlighted nucleotides in COI change amino acid from Phenylalanine in H1 (TTT) to Leucine in H2 and H3 (CTT .

Region of mtDNA marker	Population	Haplotype	1	2	3	4	5	6	7	8	9	10	11	Individuals
COI	Ns	H1	A	A	G	T	_	_	_	_	_	_	_	36
	Ns	H2	A	A	G	C	_	_	_	_	_	_	_	1
	Si	H3	G	G	A	C	_	_	_	_	_	_	_	23
tRNA	Ns	H1	T	G	G	G	T	T	A	G	G	G	A	10
	Si	H2	G	A	T	T	C	A	T	A	A	A	G	23
COII	Ns	H1	T	T	A	T	A	_	_	_	_	_	_	10
	Si	H2	C	T	A	C	G	_	_	_	_	_	_	1
	Si	H3	C	C	A	C	G	_	_	_	_	_	_	13
	Si	H4	C	C	G	C	G	_	_	_	_	_	_	4
	Si	H5	T	T	A	C	G	_	_	_	_	_	_	2
	Si	H6	C	C	A	T	G	_	_	_	_	_	_	3

Table 5.5: Genetic diversity and private haplotypes measured for the two populations of *Anthophora pauperata* across three regions of mtDNA marker.

Region of mtDNA marker	Population	No. of individuals	Private haplotype %	Gene diversity	Global gene diversity
COI	Ns	37	100	0.05	0.50
	Si	23	100	0.00	
tRNA	Ns	10	100	0.00	0.44
	Si	23	100	0.00	
COII	Ns	10	100	0.00	0.75
	Si	23	100	0.65	

Table 5.6: Genetic diversity measures for ten microsatellites loci and 7[illegible] *Anthophora* bees.

Loci	Repeat ()n	No. individual	N_a	H_o	H_e	PIC	HWD	null a.
anth24	(TC)12	78	2	0.000	0.325	0.271	NS	0.988
anth30	(CA)8	78	2	0.000	0.329	0.273	NS	0.988
anth35	(TTG)10	78	2	0.000	0.028	0.027	NS	0.405
anth40a	(CA)13	78	7	0.297	0.799	0.762	***	0.455
anth43	(TCT)19	78	4	0.178	0.501	0.393	***	0.472
anth64	(GA)15	78	3	0.066	0.374	0.307	NS	0.699
anth72	(TTC)11	78	3	0.154	0.662	0.584	***	0.623
anth73	(GAG)8	78	2	0.000	0.051	0.049	NS	0.553
anth74	(TCG)14	78	6	0.115	0.575	0.526	***	0.661
anth75	(CTT)10	78	7	0.026	0.409	0.382	NS	0.879
Average			3.8	0.084	0.405	0.357		

N_a: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: Polymorphism Information Content – value of a locus to identify polymorphism within the *Anthophora* population; HWD: deviation from Hardy Weinberg Equilibrium expectation; ***: *P*-value < 0.001; **: *P*-value < 0.01; *: *P*-value < 0.05; NS: non-significant; null a.: estimated the propability of null allele.

Table 5.7: Measures of F_{st} within and among countries for ten microsatellite loci and 78 *Anthophora* bees.

	P value		
Locus	Ns	Si	Ns-Si
anth24	_	<< 0.0001	<< 0.0001
anth30	_	<< 0.0001	<< 0.0001
anth35	0.05	_	0.2
anth40a	0.05	0.0003	<< 0.0001
anth43	0.5	<< 0.0001	0.2
anth64	0.9	<< 0.0001	0.0005
anth72	0.1	0.2	<< 0.0001
anth73	<< 0.0001	_	0.05
anth74	0.4	<< 0.0001	<< 0.0001
anth75	<< 0.0001	<< 0.0001	<< 0.0001

Ns: northwest Saudi Arabia population (Jabal Al-Loz); Si: South Sinai population (St. Katherine).

Table 5.8: Three tests for heterozygosity excess (Wilcoxon test, Standardized differences test & Sign test) under the two mutational methods in the two populations of *Anthophora pauperata*.

				Wilcoxon test		Standardized differences test		Sign test	
Pop	L	N	H_e	IAM	SMM	IAM	SMM	IAM	SMM
				p	*p*	*p*	*p*	*p*	*p*
Ns	8	35.5	0.29949	0.94531	0.02734	0.46695	0.00358	0.60068	0.01623
Si	8	39.8	0.40918	0.00781	0.01172	0.0001	0.01172	0.02101	0.03992

L: number of polymorphic loci; N: mean number of individuals sampled per locus; H_e: Hardy-Weinberg expected heterozygosity; IAM: infinite alleles model; SMM: stepwise mutation model.

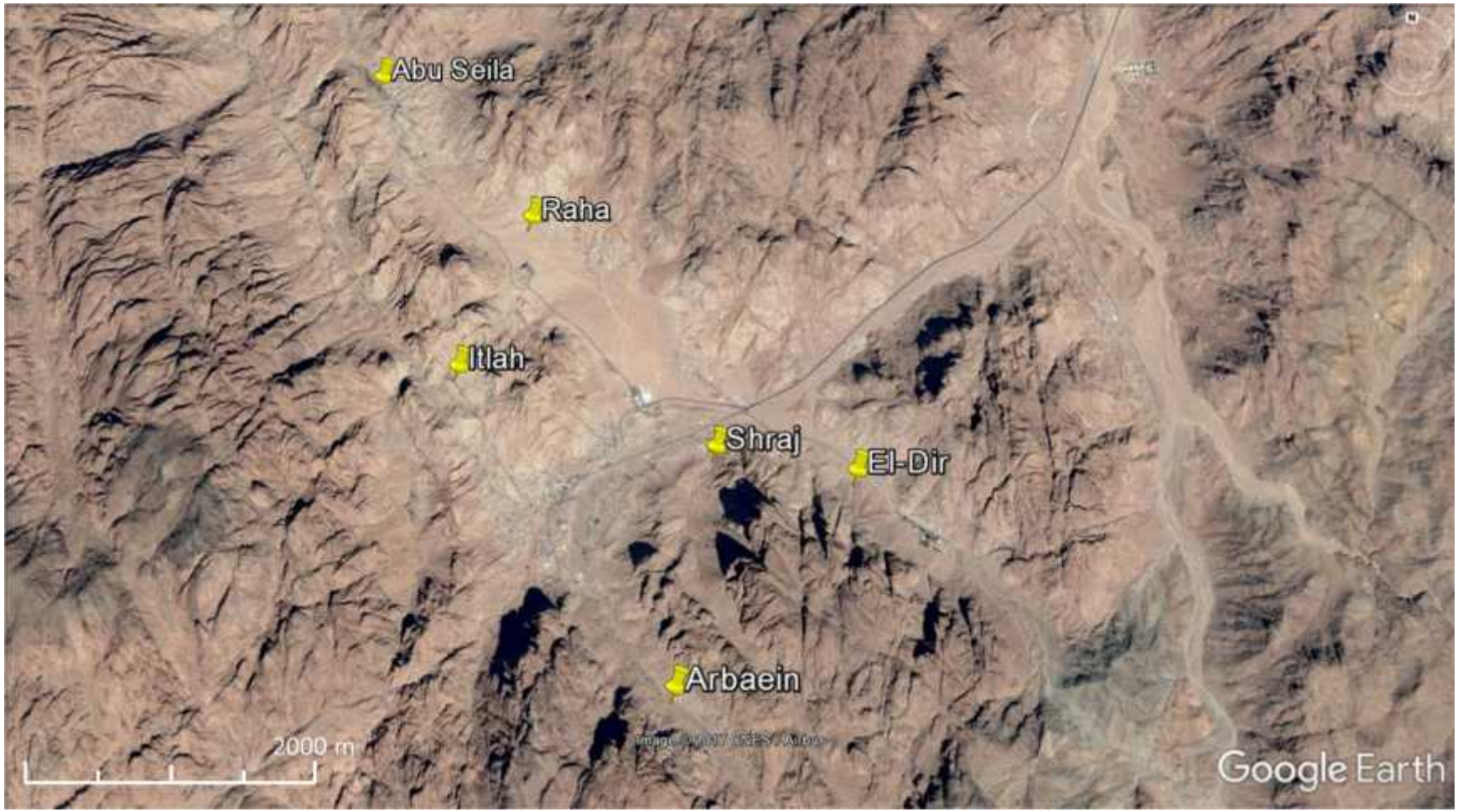


Figure 5.1: Study plots in wadis on the South of flank of Sinai, Egypt. The highest and lowest elevations from the sea level occurs in wadi Arbaein and wadi Abu Seila respectively.

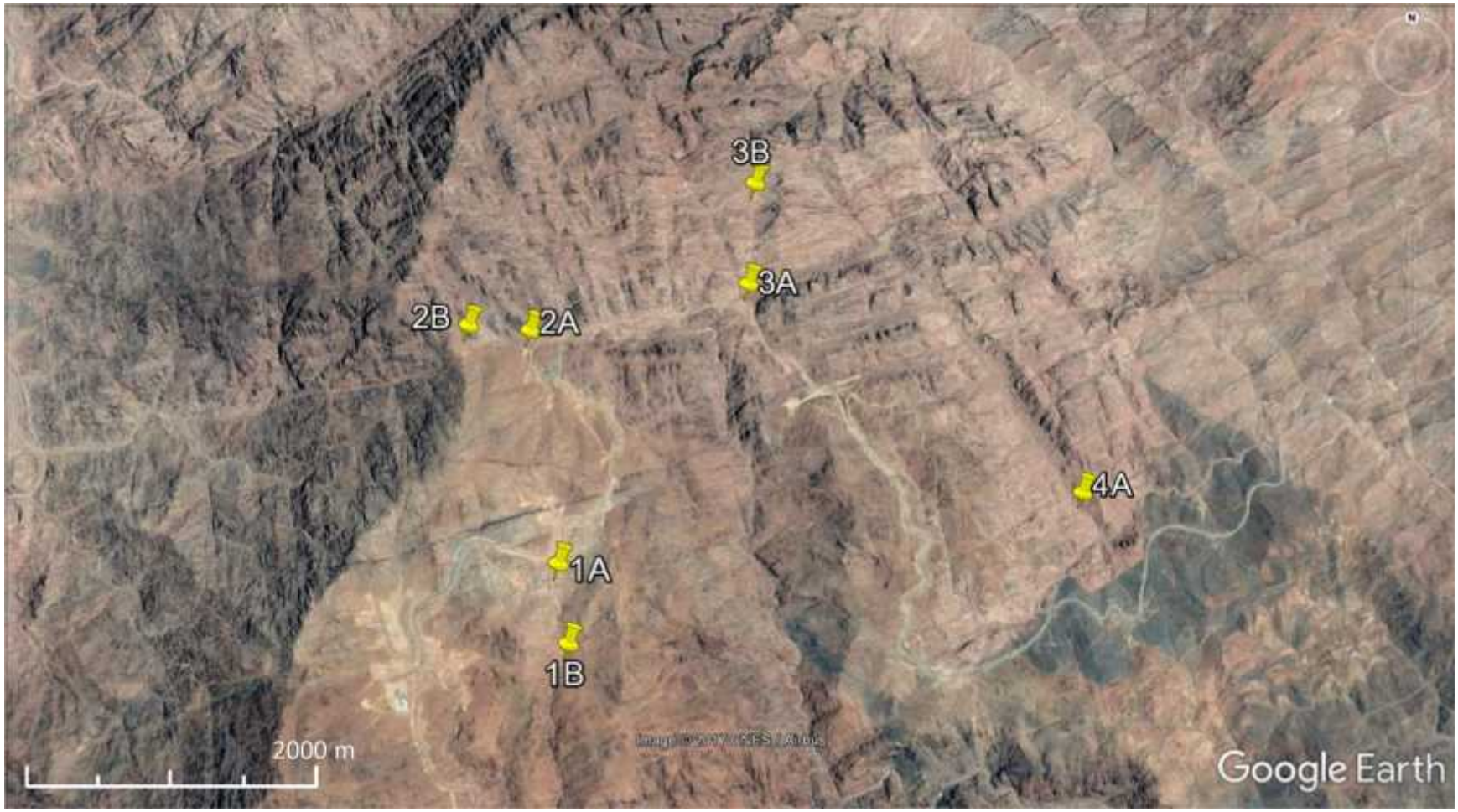


Figure 5.2: Study plots in wadis on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The highest and lowest elevations from the sea level occurs in site 1A and site 4A respectivel

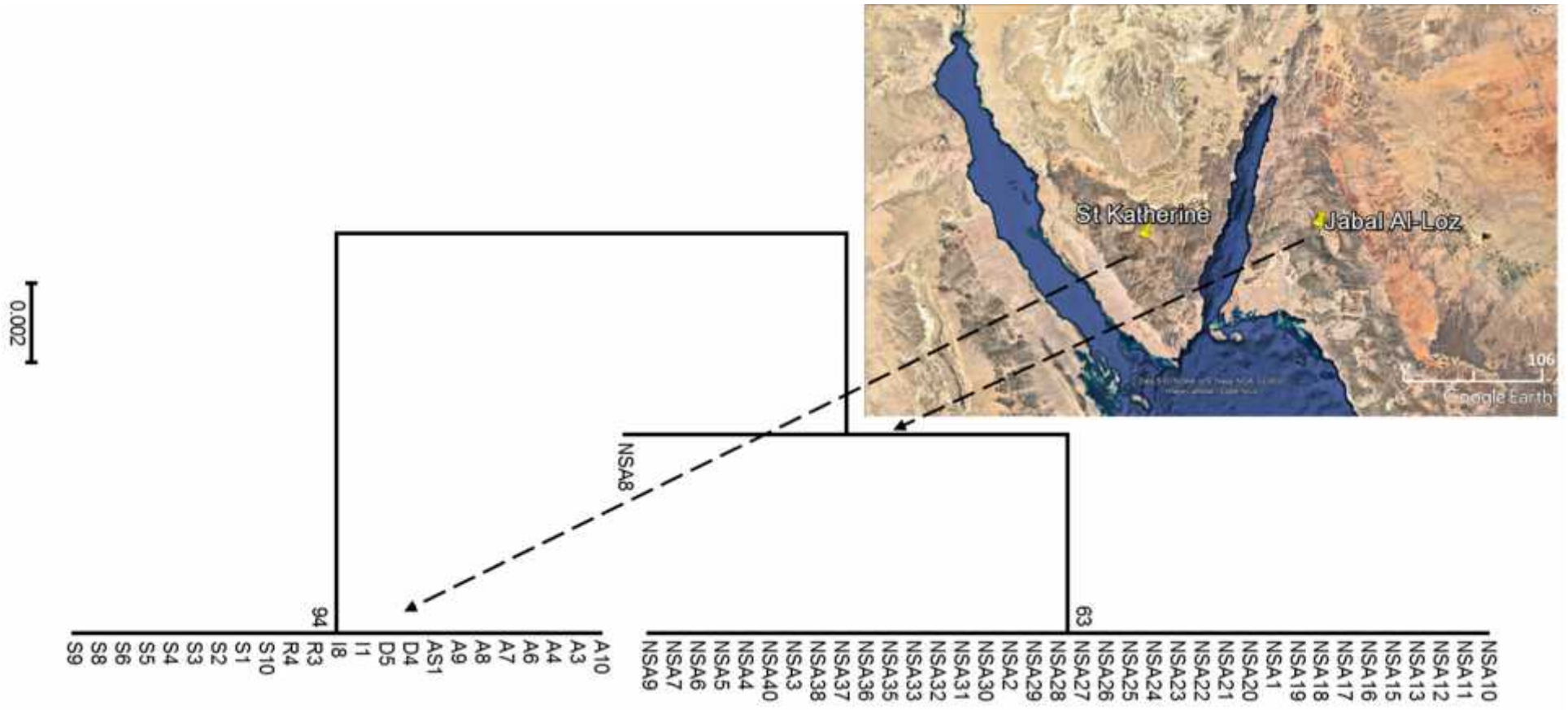


Figure 5.3: Neighbour-joining dendrogram of individual Anthophora pauperata based on mtDNA sequences (encoding a region of cytochrome c oxidase subunit I). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Two haplotypes belong to Jabal Al-Loz and one to St Katherine. Node numbers refer to Bootstrap support (1000 replicates).

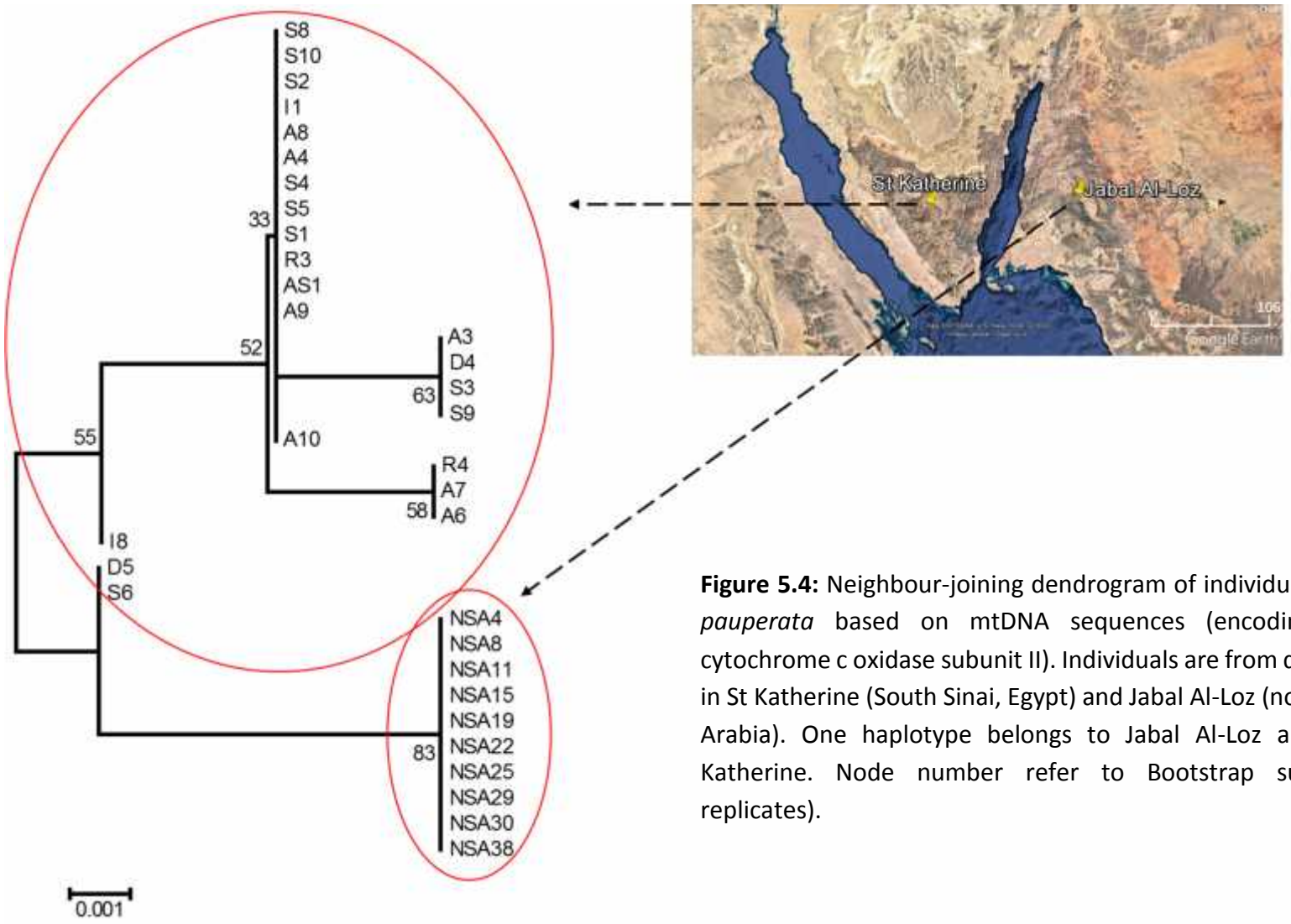


Figure 5.4: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (encoding region of cytochrome c oxidase subunit II). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). One haplotype belongs to Jabal Al-Loz and five to St Katherine. Node number refer to Bootstrap support (1000 replicates).

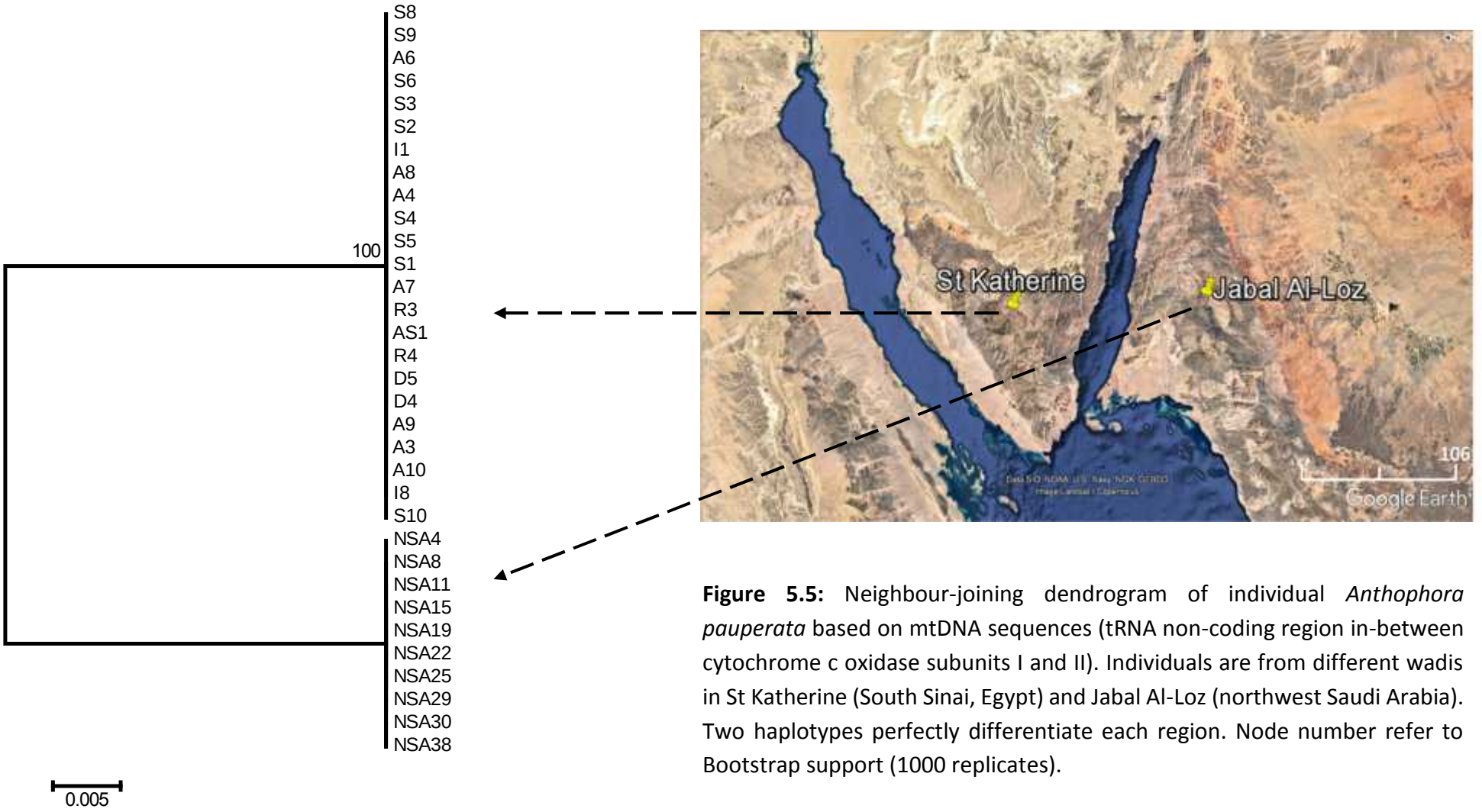


Figure 5.5: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (tRNA non-coding region in-between cytochrome c oxidase subunits I and II). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Two haplotypes perfectly differentiate each region. Node number refer to Bootstrap support (1000 replicates).

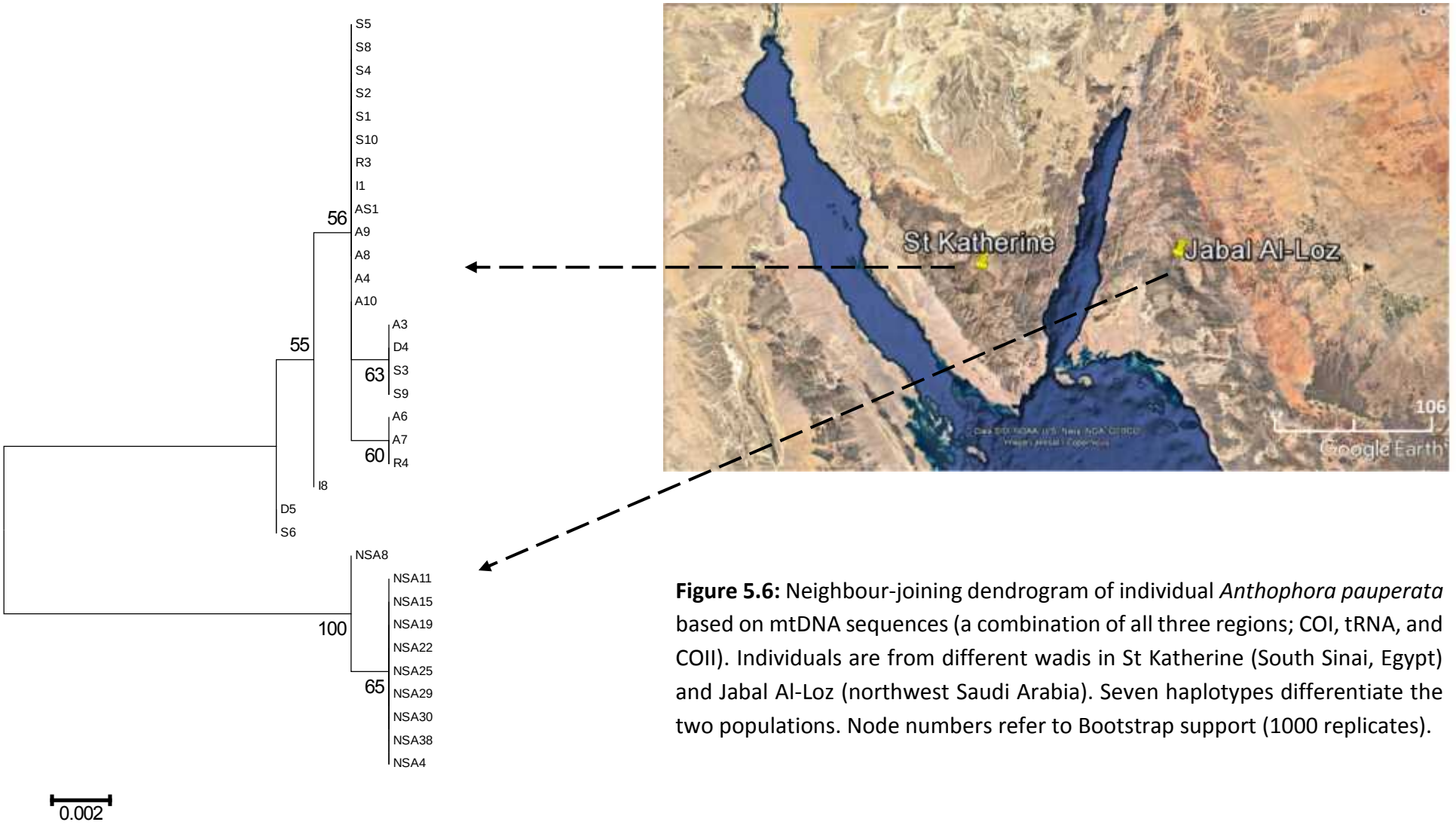


Figure 5.6: Neighbour-joining dendrogram of individual *Anthophora pauperata* based on mtDNA sequences (a combination of all three regions; COI, tRNA, and COII). Individuals are from different wadis in St Katherine (South Sinai, Egypt) and Jabal Al-Loz (northwest Saudi Arabia). Seven haplotypes differentiate the two populations. Node numbers refer to Bootstrap support (1000 replicates).

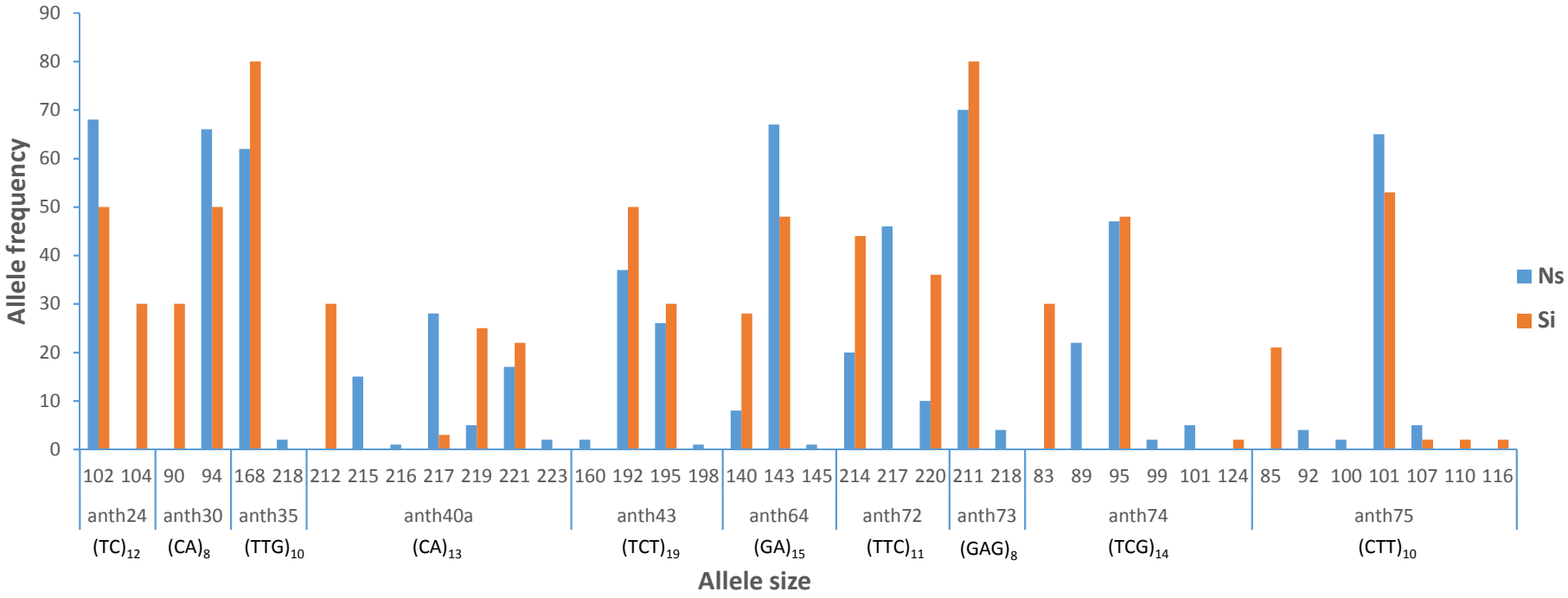


Figure 5.7: Allele frequencies per microsatellite locus across the two populations of *Anthophora pauperata*. Ns: northwestern Saudi Arabia population; Si: South Sinai population.

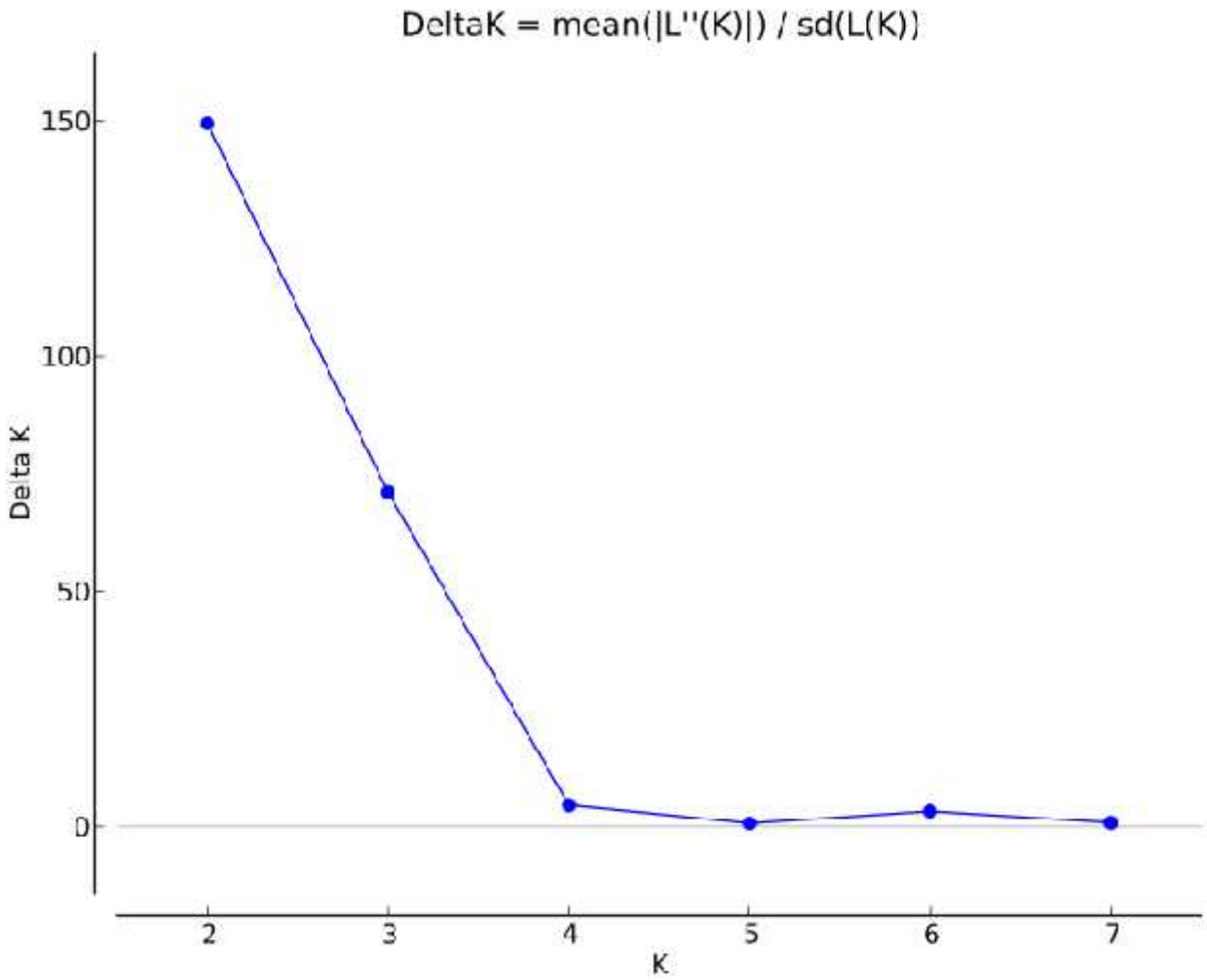


Figure 5.8: Graph representing the optimal clustering (K = from 2 to 7), from STRUCTURE HARVESTER analysis.

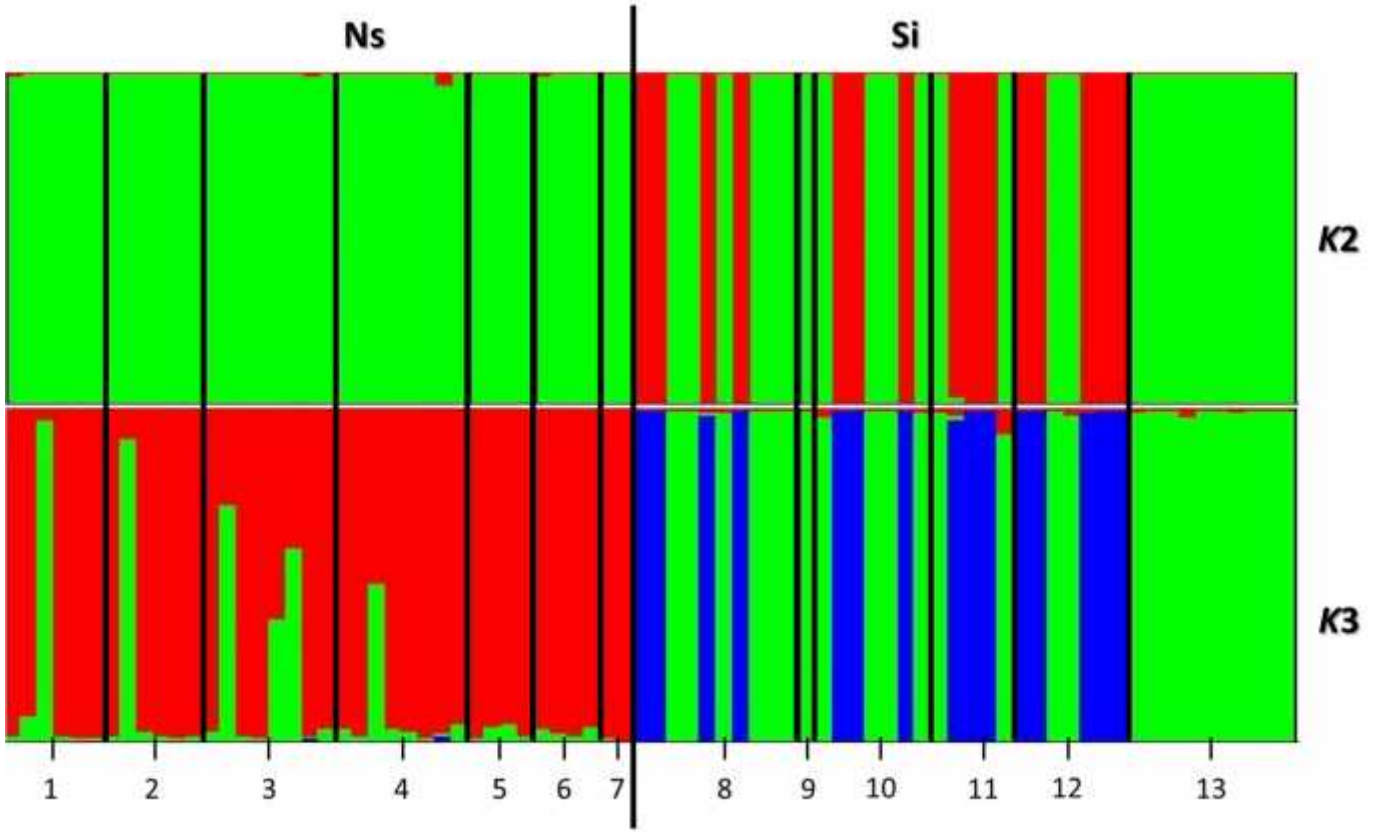


Figure 5.9: Individual assignment based on the Bayesian method under an admixture model obtained by STRUCTURE. 78 *Anthophora* bees from two populations were analysed at K = 2 and = 3 (K is the assumed number c opulations). Each subpopulation is represented by a single line and their colours correspond to the relative proportion of the inferred genome corresponding to a specific genetic cluster. The two main populations are separated by the long black line. Ns: northwestern Saudi Arabia population; Si: south Sinai population. Numbers on the X-axis represent subpopulation sites.

5.5 Discussion

The use of various molecular markers among groups of individuals is essential to comprehend the shape of the population structure. In this study, I considered maternally inherited markers from mtDNA and biparentally inherited microsatellite loci in order to study the genetic diversity and structure of *Anthophora pauperata* in the only known populations found in northwest Saudi Arabia and South Sinai. The species is the main pollinator of *Alkanna orientalis,* emerging at the same time as the flowers in early spring (Willmer *et al.*, 1994; Stone *et al.*, 1999; Zalat *et al.*, 2000; Chapter 2).

I used the maternally inherited mtDNA marker to study population genetic structure in *A. pauperata*. The three segments of the mtDNA marker were clearly differentiating the two geographically separated populations. The greatest number of variable positions lay in the COII section. The geographic region with the highest level of variability overall was south Sinai; however, the south Sinai haplotypes were shared among wadis. This is either because of recent gene flow or shared recent origin but with evidence of gene flow in-between wadis. Following the use of mtDNA, I used ten hyper-variable microsatellite loci. The microsatellite result was more or less consistent with the mtDNA, again differentiating the Saudi from the South Sinai population. Again, the greatest degree of genetic diversity was found within the South Sinai population.

The population differentiation is consistent with geography, since northwest Saudi Arabia and South Sinai are isolated by the Gulf of Aqaba, the Red Sea to the south and the deep valley of Wadi Araba to the north, which likely restricts gene flow between these regions. *Anthophora pauperata* is characterised as a relatively specialized bee, and is therefore suggested to have considerably less genetic variation within populations and more genetic differentiation among populations; the absolute value of the variation

is greater than the mean for generalists (reviewed by: Futuyma *et al.*, 1988; Zayed *et al.*, 2005; Li *et al.*, 2014; Hung *et al.*, 2017).

This result is also consistent with the long-movement distances of *Anthophora* bees: I showed in chapter 3 that *Anthophora* bees in northwest Saudi can travel as far as 4 km in spite of the mountain barriers. However, most bees do not move this far, which likely explains the low variation and mixing of genes between wadis within each population. Female *Anthophora* may mate only once during their lifetime, or perhaps once per nest over several nests (Gilbert *et al.*, 1999). On the assumption that migration occurs, a female could mate several times in a particular location before moving to a different locality. Asymmetry in the migration rate between populations might explain phenomena such as genetic uniformity in the Saudi population *versus* diversity in the South Sinai population.

The possible restriction of gene flow in South Sinai might result from the movements of *Anthophora* being more affected by natural landscape barriers and low temperatures, making long-distance flight more costly even for this extremely effective thermoregulator (Mahmoud *et al.*, 2008). This may not be an issue in the Saudi population, where bees were clearly able to make effective movements among wadis.

There is another possible explanation for the observed results, which is that gene flow is linked to the foraging behaviour of *Anthophora*. There are three types of males with different behavioural strategies for mating, (in the south Sinai population: Willmer *et al.*, 1994; Gilbert *et al.*, 1999; Stone *et al.*, 1999; in the northwest Saudi population: chapter 3). Some males are territorial, guarding a set of *Alkanna* plants; others have a sit-and-wait strategy, saving the high energy costs of constantly flying around the patches of *Alkanna*; and some are floaters, adopting a strategy of moving widely across

the wadi floors, entering the guarded areas of a series of territorial males. Females forage more widely on *Alkanna* plants encompassing several male territories near or including their nest location, perhaps travelling larger distances when they start digging a new nest. It is possible that the long-distance movements I found in Saudi were associated with the completion of one nest and the starting of another. I saw females interact with floater males several times (chapter 3), although most of their interactions were with territorial males. It is possible that the northwest Saudi females might mate more frequently with floater males than in Sinai, or move further among nests, resulting in a difference in overall gene flow and genetic variation between these populations.

With K=2, the microsatellite markers showed many shared alleles between the Saudi and South Sinai populations, with a single private allele in South Sinai (Fig. 5.9). However, if K=3, the structure showed a significant level of differentiation between th two populations, with some individuals from Saudi sharing the same allele as the South Sinai population (Fig. 5.9). Despite sharing a large number of alleles (green in Fig. 5.9), there was a sufficient number of private alleles to distinguish the two populations (Fig. 5.7), and the variation in allelic frequencies also differentiating these populations

The differences found in population structure between the mtDNA and microsatellite markers can be explained by females dispersing smaller distances than their male counterparts. This is because mtDNA is only inherited through the maternal line and its evolution is therefore based only on female-mediated processes). It is also possible that the rate and pattern of mutation differs between mitochondrial genes and microsatellite loci. Furthermore, mtDNA has an effective population size that is four-time less in comparison to nuclear genes because of its maternal inheritance (Birky *et*

al., 1983; Desalle *et al*., 1987). Consequently, shifts in population allele frequencies may occur faster in mtDNA markers than in microsatellites.

The presence of recent bottlenecks was tested by examining levels of heterozygosity relative to allelic diversity. Bottlenecks are expected to reduce the number of alleles but not reduce heterozygosity to the same extent. I evaluated the data under two models of mutation – the stepwise mutation model and the infinite alleles model. Microsatellites are more likely to evolve in a way modelled by SMM than IAM, and the latter predicts a lower equilibrium heterozygosity (Luikart *et al.*, 1998). Results using both methods suggest that the South Sinai population may have been subjected to a recent severe bottleneck because there is a significant probability of recent population reduction under both mutational models in all three tests, while the northwest Saudi population only showed signs of a bottleneck under one model. However, specialists are predicted to be more sensitive to alterations in habitats or resources than generalists, and thus more susceptible to suffering repeated population bottlenecks (Li *et al.*, 2014). The limited indications of bottlenecks in the Saudi population might suggest better habitat quality or minimal anthropogenic interference.

Usually conservation planning should give higher priority to areas with high rates of genetic variants (Driscoll, 1998) to preserve as much genetic variation as possible, followed by populations that face damage caused by human activities. In general, humans are the main cause of threats to biodiversity (Pimm *et al.*, 2014). From our study the South Sinai population should receive a higher conservation priority. This is because it has a higher level of variation. The data also suggest that it has been exposed to more recent severe bottlenecks; it definitely has had more human impacts, from Bedouins settling the region over the last 1500 years, and the late twenti -century disruption

of the Israeli invasion and modern tourism. There is evidence that the Saudi population has also suffered from recent bottlenecks, and hence it is also of conservation concern.

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Chapter 6: General summary

This thesis delivers the first detailed observations of the daily pattern of *Anthophora pauperata* bees, the main visitor of *Alkanna orientalis* at Jabal Al-Loz in the north-western part of Saudi Arabia, and compares these with previous studies in south Sinai in Egypt. It also presents the first characterisation of genetic diversity and population structure of the only two known populations of these bees at Jabal Al-Loz and south Sinai, as well as the genetic structure over a large geographic scale of its main food resource, the plant *Alkanna orientalis*. These studies together provide an overview from both genetic and ecological perspctives, which together allow a range of conclusions to be drawn.

6.1 From an ecological point of view

6.1.1 Is Anthophora pauperata the main pollinator of Alkanna orientalis?

Intensive observations of the visitors of *Alkanna* flowers in different wadis at Jabal Al-Loz in the north-western part of Saudi Arabia enhance our knowledge as to which species have the most significant relationships with *Alkanna* flowers. Our results confirmed that *Anthophora pauperata* is the most common visitor to *Alkanna orientalis* and the only efficient pollinator per flower visit, consistent with earlier findings in South Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Stone *et al.*, 1999).

The season of both insect and plant in the Saudi populations perhaps starts slightly earlier than those in South Sinai. A variety of patterns were found among sites in term of seed production, which might be due to the distinctive features of each site, such as plant density, or other factor; however, the overall probability of pollination was

decreasing with time, consistent with the decreasing bee density, indicating that the seasons for both the plant and bee were finishing simultaneously.

6.1.2 Anthophora pauperata movements

Close observation of the flowers' visitation in the north of Saudi indicates that *Anthophora* visit *Alkanna* flowers non-randomly. *Anthophora* prefer to visit plants with lots of flowers, which mean lots of nectar and pollen resources. Such strategies of large floral displays mean high level of rewards, a decreased cost of traveling time and more return visits. *Alkanna* flowers change colour when pollinated, indecating reduced production of nectar and lack of pollen resource (Nuttman *et al.*, 2006). Hence, *Anthophora pauperata* probably have the ability to learn from experience to visit more rewarding flowers by correlating colour with reward size, as in bumblebees (Cartar, 2004; Essenberg *et al.*, 2015).

Long-distance movements were recorded in the Saudi population: females (but not males) were recorded traveling long distances between connected and also between unconnected wadis. Such long-distance movements may result from high competition at a particular site, the search to find a new nest site, or both. However, my result from a molecular point of view indicates that over the long term there is continuity of gene flow for both bee and plant over long distances irrespective of the harsh landscape and high mountain barriers of the region. Such a finding is not compatible with the earlier study (Wolff *et al.*, 1997) in south Sinai, which proposed that gene flow of the plant essentially occurs only through seed movement during periods of flash-flooding, and is unlikely to occur via pollen movement by bees. Wolff *et al.* (1997) thought the lack of bee-mediated gene flow was the result of the territorial behavioural

of the bees coupled with the high mountains barriers that isolate the wadis by hindering movement between wadis.

6.1.3 The foraging and mating patterns of Anthophora pauperata

The current study of the daily pattern of behaviour of *Anthophora pauperata* fulfils our desire to understand more of its entire life style and relationships with other species, including its main food resource. In Saudi Arabia the bees showed similar behaviour to those in South Sinai (Willmer *et al.*, 1994; Gilbert *et al.*, 1996; Wolff *et al.*, 1997; Stone *et al.*, 1999; Nuttma *et al.*, 2006). Both males and females mainly or exclusively visited one plant, *Alkanna orientalis*. Females showed bimodal diurnal foraging activity, with peaks in the early morning and late afternoon, separated by nesting activity in the middle of the day. Males showed a unimodal pattern of activity, arriving at the time when females were about to abandon the foraging site in the morning, and leaving just after females returned back to the foraging site.

In most studied cases, the daily pattern of insect foraging is considered to change in response to the availability and accessibility of food resources, daily variations in microclimate, the cost of thermoregulation, and the predation threat (Armbruster *et al.*, 1990). The bimodel activity of fema *Anthophora pauperata* was not based on high ambient temperature at the middle of the day because they have an efficient thermoregulation system and so activity is more or less independent of ambient temperature (Stone *et al*., 1999). The ambient temperature at the middle of the day was not different from that of the late afternoon, and a few females continued foraging activity through the midday lull. The threat of predation does not seem to drive the bimodal activity in this system. The explanation for the bimodal activity that best fits the

facts is that female *Anthophora* are responding to resource availability: at midday *Alkanna* flowers have insufficient nectar and zero pollen (Stone *et al*., 1999), and thus foraging will not be worthwhile. It would be much better to use this time to dig nests.

The unimodal pattern of diurnal activity of males of *Anthophora pauperata* is obviously connected with their territorial behaviour, and they show many types of behaviour through the day: patrolling, foraging, resting, mating interactions with females, and aggressive interactions with other territorial males and some other species. The aggressive behaviour was much stronger with other territorial males than with other insects, and occurred at times when females were absent. In contrast, females mostly forage peacefully together, with occasional female-female aggressive interactions as in South Sinai, interpreted by Gilbert *et al.* (1999) as suggesting that each male has 'its' females foraging in his territory, or possibly a set of related females share the same resources for nesting and foraging. It is also may relate to pollen competition, since most such interactions happened in the late afternoon when pollen presentation peaks (Stone *et al.*, 1999).

Different interpretations have been put forward to explain the apparently non-overlapping diurnal activity patterns of the sexes of *Anthophora pauperata*. Willmer *et al.* (1994) described it as an effort by males to guard food for females while the latter were digging a new cell, particularly so that females could collect pollen after the males have disappeared. They thought, therefore, that male behaviour was not to obtain matings, but instead should be regarded as investment in their own offspring. An alternative interpretation arose by Gilbert *et al.* (1999) due to the fact that such a system was clearly cheatable: why perform such energetically costly territorial activity when matings could be secured without it?

In Saudi Arabia, few matings were recorded by a non-territorial male. Interestingly, once I observed a mating by a non-territorial male that was noticed by the territory-holding male: this resulted in a long-distance chase and the same female re-mated by the territorial male. Therefore, the essential point is the mating behaviour of females (Gilbert *et al.*, 1999). If a female either mates just a few times, each time she nests, or mates only once soon after eclosion, then it seems reasonable to interpret the function of the territoriality system as one of using attraction to floral resources as a way of searching for new unmated females – i.e. resource-defence polygyny (Sakai *et al.*, 2001). It has been noticed frequently in both Sinai and Saudi that although supposedly motivated to gain matings, males hover around some females but leave them untouched. Furthermore, territorial males in Saudi Arabia were frequently noticed to settle for relatively long periods on top of grasses or tree leaves, possibly a style of inspection for emergence sites.

Further supporting the work of Gilbert *et al.* (1999), our study in Saudi Arabia in early March showed that at one site the territorial behaviour did not begin until a week after observations started, when the number of females had noticeably increased. I interpret this as the costs of territoriality making it unreasonable at the beginning of the season when males were much commoner than females. In some more open sites, overall bee densities were low, and here too there was no evidence of territoriality. I interpret these data as suggesting that territoriality only occurs where flower, bee and especially female densities are high enough. It may be that all males can adopt the 'floater' strategy when female densities are low, but when females become commoner, males fight to become territory holders with the losers either remaining nearby as non-holders, or roaming widely as floater males.

6.2 From a molecular point of view

6.2.1 In the plant

Plant conservation is a fundamental element of the conservation of ecosystems and ecosystem function, and genetic studies are an important contributor to the discussions that inform decisions on giving priority to populations most at risk. From data for 4 plant species, Hamrick *et al.* (1990) suggested that endemic species are genetically less diverse than widespread species, and more susceptible to bottlenecks due to having smaller populations with more limited distributions. This finding agrees with our interpretation of the molecular results on *Alkanna orientalis*, which shows population genetic structure at different spatial scales in the eastern Mediterranean and Middle East regions. The genetic structure at such a large geographical scale showed a spatial pattern which might be the consequence of ancient processes of a series of expansions and reductions; whereas at a smaller geographical scale there was also evidence of spatial diversity, perhaps emphasizing more recent, localised events of population extinction and recolonization from adjacent areas, or fluctuations in population size associated with chance genetic drift when population size is small.

Different types of genetic markers can generate a rich variety of data on genetic diversity and structure within individual species (Geburek, 1997). The differences that I observed between the two different types of molecular loci in *Alkanna*, one of which is biparentally inherited and the other maternally inherited, likely reflect the differences in the rate of gene flow of pollen *vs.* seeds, as well as differences amongst the chosen loci in their underlying rate of mutation.

6.2.2 In the bee

Bees are an essential element of ecosystems, playing an invaluable role as pollinators of wild plant and crops (Klein *et al.*, 2007; Kremen *et al.*, 2007). Many studies have found that bees are susceptible to extinction for genetic reasons (Franck *et al.*, 1998; Zayed, 2009; Pot *et al.*, 2010). Genetic studies are thus able to provide precious data for conservation management. I have used both maternally inherited markers from mtDNA and biparentally inherited microsatellite loci to study the genetic diversity and structure of *Anthophora pauperata* at the only two known populations found in northwest Saudi Arabia and South Sinai.

I found spatial pattern in the genetic structure over a large geographical scale among populations. Such a finding was predictable since the two populations are disconnected because of the Gulf of Aqaba, which definitely restricts or prevents gene movement between these regions. Such a result was expected in term of the barriers and the distance causing populations to become genetically distinct through localised selection or random genetic drift. Populations that are genetically diverged from one another may react differently to environmental factors, and therefore might be considered as distinct entities in terms of their conservation (Davis *et al.*, 2010).

On the other hand, less variability has been seen over small geographical scales within populations. These mostly occurred in the South Sinai population, although the South Sinai haplotypes were shared among wadis. This may be the result of recent gene flow or a shared recent origin but with signs of gene flow among wadis. However, the higher variability found in South Sinai might be a result of the more restricted area, and/or low temperatures causing movements to be more costly. Although not obvious

from the data, it is possible that males with different mixes of mating strategies might be the cause of the differing levels of variability between the two regions. Males with the more wide-ranging floater strategy (who forage and search for mates across a wide area including the defended areas of territorial males) might be more common in the Saudi population, leading to greater genetic homogeneity. Overall, *Anthophora pauperata* is considered to be a comparatively specialized bee, and therefore should be more likely to have limited genetic variation within populations and more genetic differentiation among populations than generalist species (reviewed by: Futuyma *et al.*, 1988; Zayed *et al.*, 2005; Li *et al.*, 2014; Hung *et al.*, 2017).

6.3 Are the ecological findings consistent with the molecular findings in the north western part of Saudi Arabia?

Population genetic structure is a result of combined actions of molecular and ecological factors (Loveless *et al.*, 1984; Hughes, 1998; Duminil *et al.*, 2007). In plant-pollinator interactions, ecological factors of productivity and dispersal are essential in shaping the population genetic structure. The current results from the ecological work are clearly consistent with the molecular evidence found in both bee and plant. The molecular results at small spatial scales at Jabal Al-Loz and in South Sinai showed evidence of the mixing of genes among wadis for both plant and bee. Such results are in complete agreement with the ecological studies of bee behaviour, since female *Anthophora pauperata* were documented as able to travel long distances between wadis in the Saudi population. Such long-distance movements by *Anthophora* distribute its own genes and those of the plant through transferring pollen among wadis.

6.4 Conclusion

Conservation schemes should give a greater attention to areas with high numbers of genetic variants, followed by populations under excessive threat from human activity. From a plant point of view, the eastern Mediterranean should receive the highest conservation priority, as a refugial region and due to the long and destructive impact of humans, followed by southwest Saudi Arabia, which is a distinct population and also experiences the unfavourable impacts of human population growth. Regions that may have been exposed to the Pleistocene glaciation may also be important in conservation terms if they are diverse as a result of having contained refugial populations.

From the bees' point of view, the high level of genetic differentiation suggests that the two populations are mostly isolated, and therefore population demography should be determined in response to local factors rather than by immigration. The low genetic diversity found in north Saudi indicates either a very small population or a recently colonized one. Thus the South Sinai population should receive greater conservation priority as it has a higher level of genetic variation and is exposed to more recent severe bottlenecks. The Saudi population may well also have suffered from recent bottlenecks.

Generally speaking, humans constitute the main driver of threats to biodiversity (Pimm *et al.*, 2014). Solitary bees have as significant a role in the earth's ecosystems as that of honeybees and other bee species. It plays a significant role in terms of plant pollination. From the specimens collected in the northwest and southwest of Saudi Arabia, there was an obvious difference in bee composition. Most bee species occurred

at Jabal Al-Loz in the northern dry desert area, even though most flower species occurred in the south, a moist and green area. This may be the result of human impacts that are clearly found in the agricultural intensification and the associated misuse of pesticides in the south. Much of the land is subjected to plowing which destroys underground bee nests. Cooperation across different government departments is needed in order to define the importance of different bee species to the general public, and farmers in particular, and to establish a proper conservation scheme for bees and their host plants, for example, by reducing habitat loss and producing bee-friendly gardening habitats.

There are limited sources of information on plant-pollinator interactions in arid areas (e.g. Gómez *et al.*, 1996; Pan *et al.*, 2012). Further investigations are neec especially, for species that are specialists and endemic to limited regions. Combined ecological and molecular studies are extremely useful here to understand the level of risk of biodiversity loss, and to develop suitable strategies for conservation. Some of the bees collected from the north of Saudi Arabia were new to science and will be studied in detail for conservation. There may be other populations of my study species in existence in Saudi Arabia, and hence several places need to be revisited, such as Jabal Al-Ward and Al-Shyati, to search for the main pollinator of *Alkanna*. Further places should be visited along the mountains of the western side of Saudi and Oman.

6.5 References

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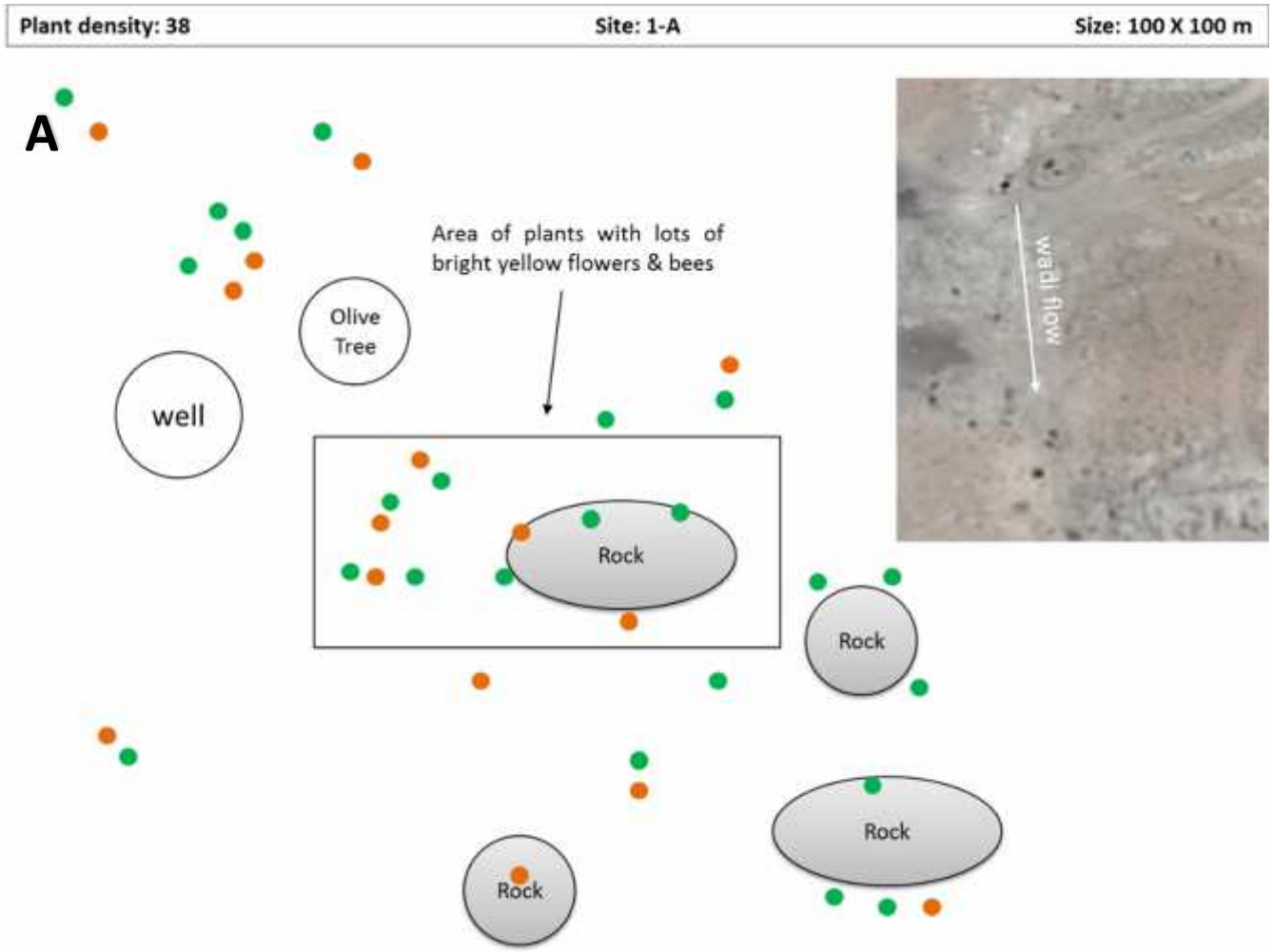
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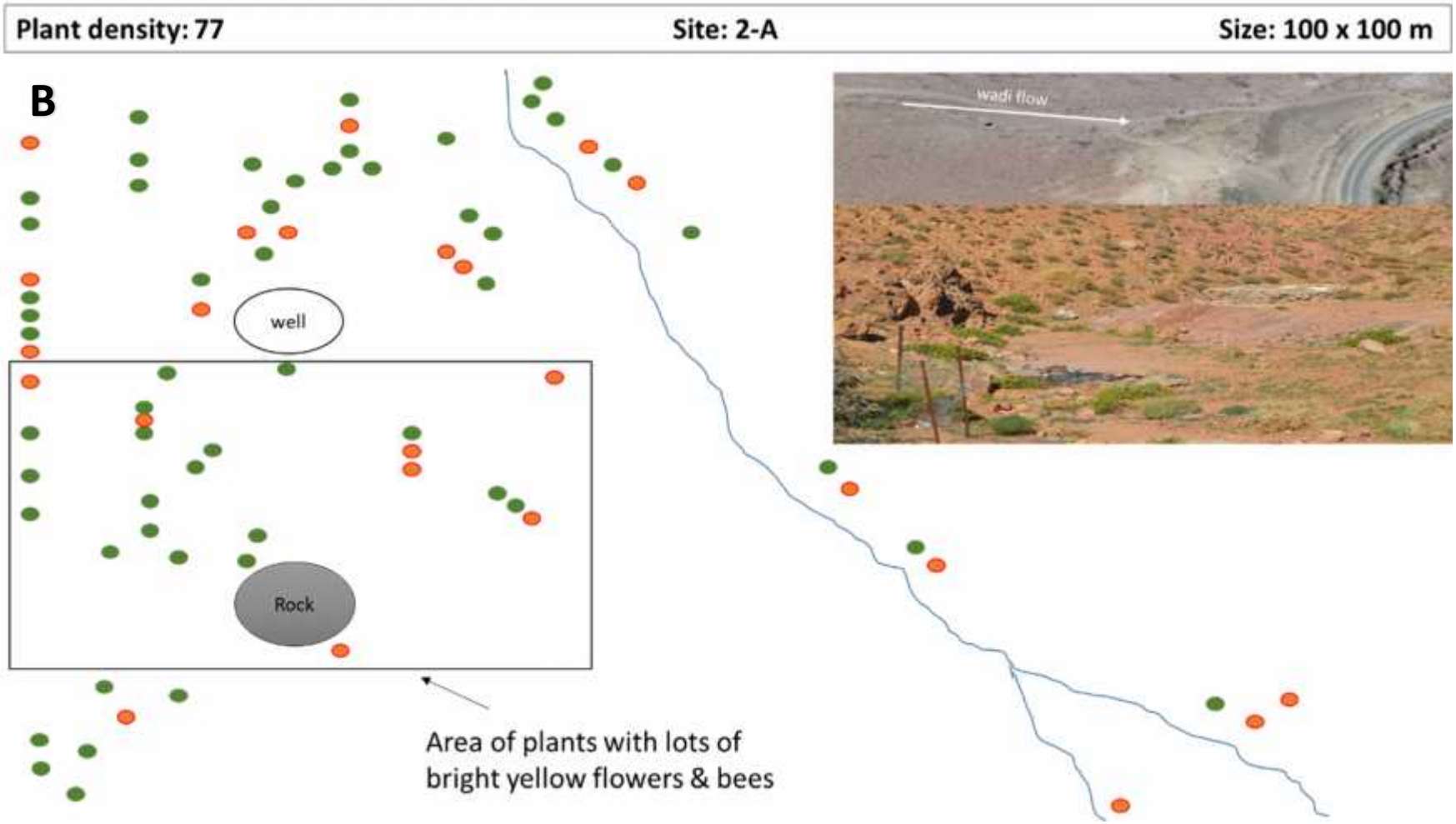
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Supplementary

Markings						First check				Second check				Third check			
Date	Wadi	Site (100x100m)	Marked colour	Sex	Marked bees	Date	Recorded Bees	Movement	Distance/m	Date	Recorded Bees	Movement	Distance/m	Date	Recorded Bees	Movement	Distance/m
2-3/03/2015	1	A	White	M	9	11/03/2015	3	_	0	21/03/2015	3	_	0	01/04/2015	3	_	0
			Red	F	15	11/03/2015	7	_	0	21/03/2015	4	1	2150	01/04/2015	3	_	0
		B	Yellow	M	6	11/03/2015	2	_	0	21/03/2015	2	_	0	01/04/2015	2	_	0
			Green	F	10	11/03/2015	4	_	0	21/03/2015	3	_	0	01/04/2015	2	_	0
2-3/03/2015	2	A	Yellow	M	21	11/03/2015	5	_	0	21/03/2015	4	_	0	01/04/2015	3	_	0
			Blue	F	14	11/03/2015	5	_	0	21/03/2015	4	_	0	01/04/2015	2	_	0
		B	White	M	2	11/03/2015	1	_	0	21/03/2015	1	_	0	01/04/2015	1	_	0
			Green	F	4	11/03/2015	2	_	0	21/03/2015	1	_	0	01/04/2015	0	_	0
2-3/03/2015	3	A	White	M	15	11/03/2015	6	_	0	21/03/2015	4	_	0	01/04/2015	3	_	0
			Red	F	27	11/03/2015	6	1	2150	21/03/2015	5	_	0	01/04/2015	3	1	650
		B	Green	M	5	11/03/2015	0	_	0	21/03/2015	0	_	0	01/04/2015	0	_	0
			Yellow	F	10	11/03/2015	2	_	0	21/03/2015	1	_	0	01/04/2015	1	_	0
4-5/03/2015	4	A	Blue	M	6	12/03/2015	3	_	0	22/03/2015	2	_	0	02/04/2015	2	_	0
			Yellow	F	19	12/03/2015	7	_	0	22/03/2015	5	_	0	02/04/2015	3	1	3750
4-5/03/2015	5	A	Green	M	3	12/03/2015	1	_	0	22/03/2015	1	_	0	02/04/2015	1	_	0
			White	F	7	12/03/2015	2	_	0	22/03/2015	1	_	0	02/04/2015	1	_	0
		B	Blue	M	5	12/03/2015	2	_	0	22/03/2015	2	_	0	02/04/2015	2	_	0
			Red	F	6	12/03/2015	2	_	0	22/03/2015	2	_	0	02/04/2015	1	_	0
4-5/03/2015	6	A	Yellow	M	3	12/03/2015	1	_	0	22/03/2015	1	_	0	02/04/2015	1	_	0
			Green	F	15	12/03/2015	8	_	0	22/03/2015	6	_	0	02/04/2015	3	_	0
		B	White	M	2	12/03/2015	1	_	0	22/03/2015	1	_	0	02/04/2015	0	_	0
			Red	F	12	12/03/2015	3	_	0	22/03/2015	2	_	0	02/04/2015	1	_	0
4-5/03/2015	7	A	Green	M	15	12/03/2015	3	_	0	22/03/2015	2	_	0	02/04/2015	1	_	0
			Yellow	F	16	12/03/2015	4	_	0	22/03/2015	3	_	0	02/04/2015	1	_	0
Total number of observed bees					247	_	80	1	_	_	60	1	_	_	40	2	_

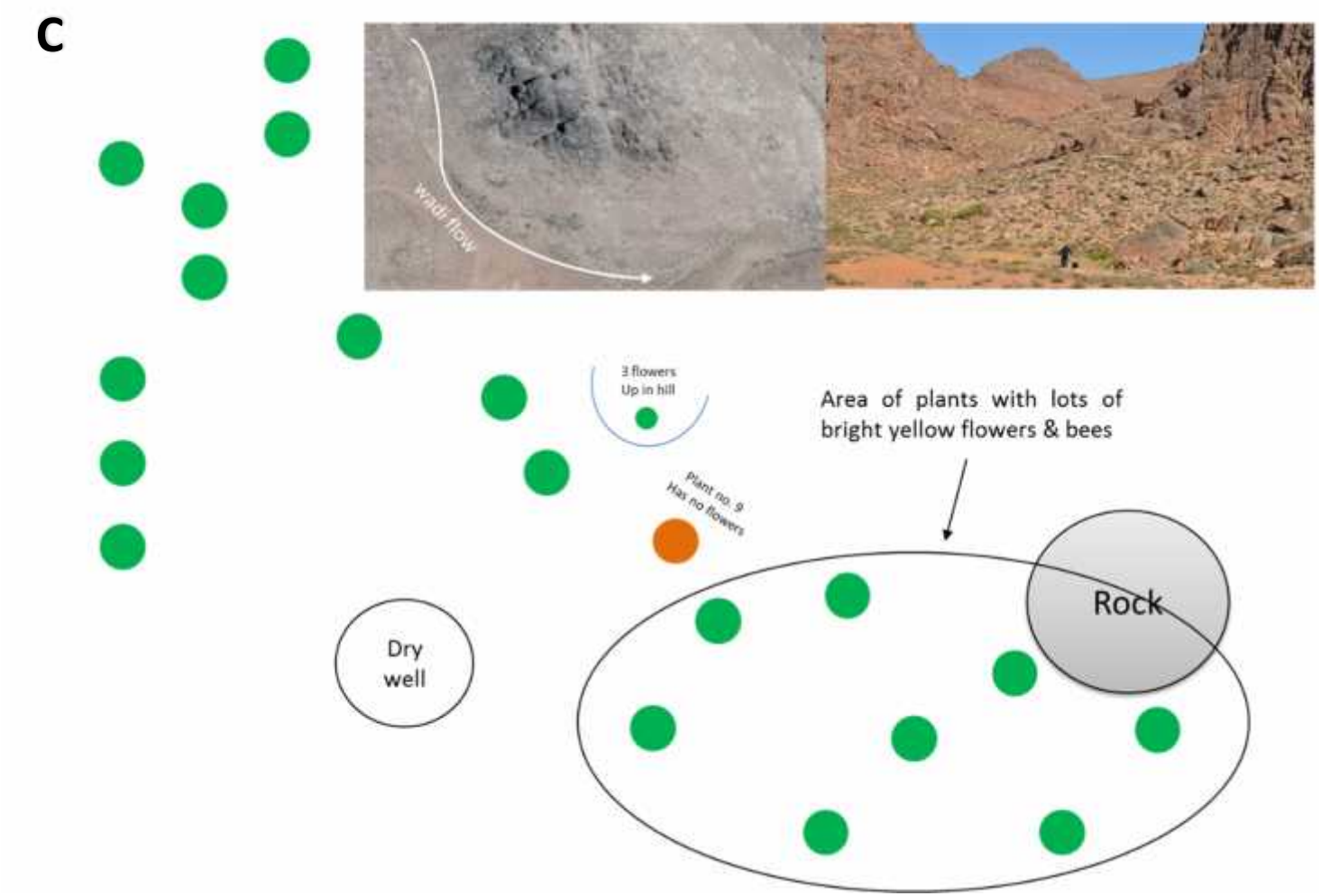
Table S1: The residence and movement of all marked *Anthophora pauperata* bees at Jabal El-Loz in the northern part of the western highlands of Saudi Arabia during March-April 2015. Each site was 100 X 100 m, with at least 500 m between sites within the same wadi. Bees were marked by different colours and numbers and were checked at regular intervals.

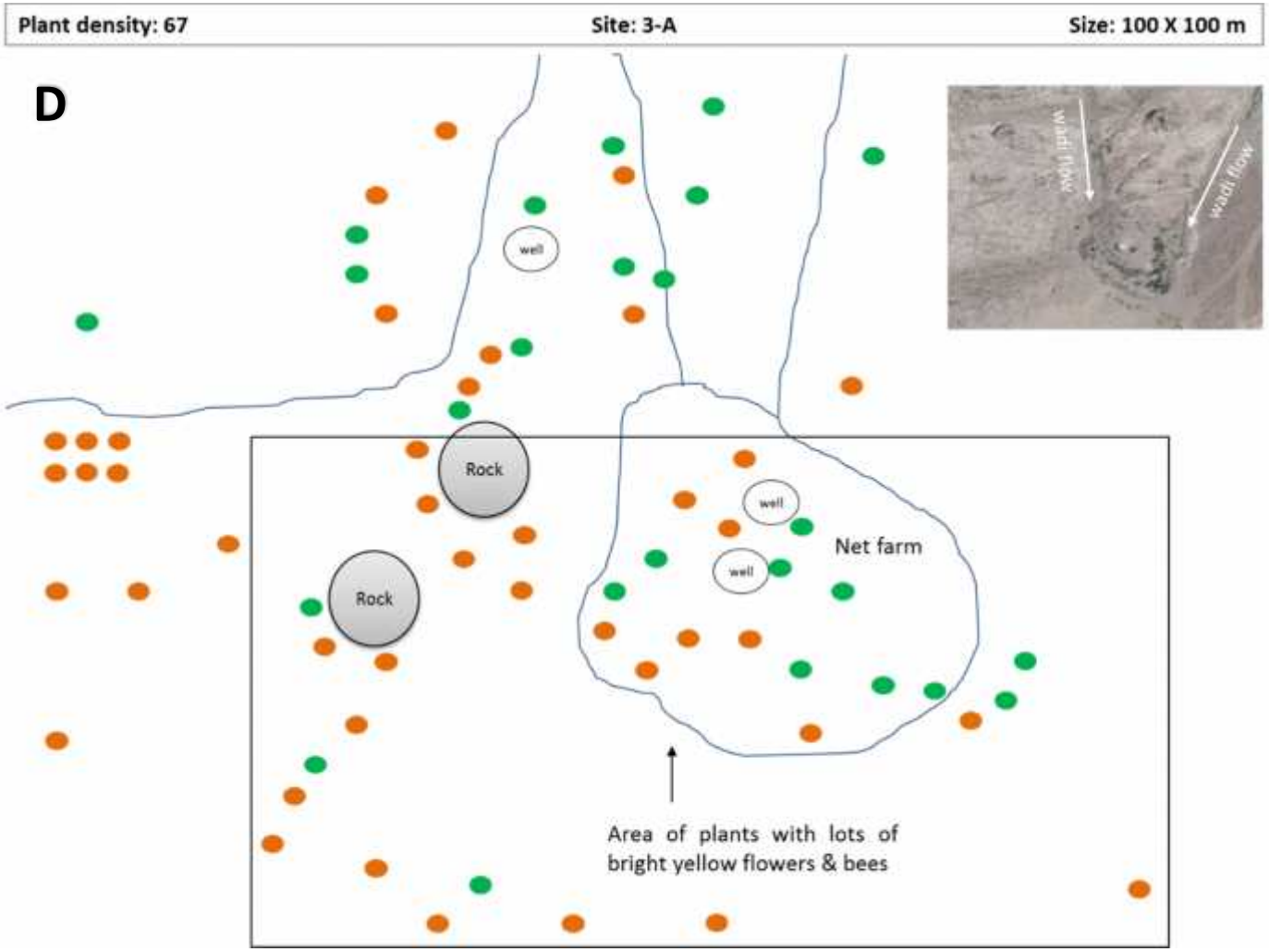

Plant density: 38
Site: 1-A
Size: 100 X 100 m
A
Area of plants with lots of bright yellow flowers & bees
Olive Tree
well
Rock
Rock
Rock
Rock
wadi flow


Plant density: 77
Site: 2-A
Size: 100 x 100 m
B
well
Rock
wadi flow
Area of plants with lots of
bright yellow flowers & bees

Plant density: 21	Site: 2-B	Size: 100 X 100 m

C




Plant density: 67
Site: 3-A
Size: 100 X 100 m
D
well
Rock
Rock
well
well
Net farm
wadi flow
wadi flow
Area of plants with lots of bright yellow flowers & bees

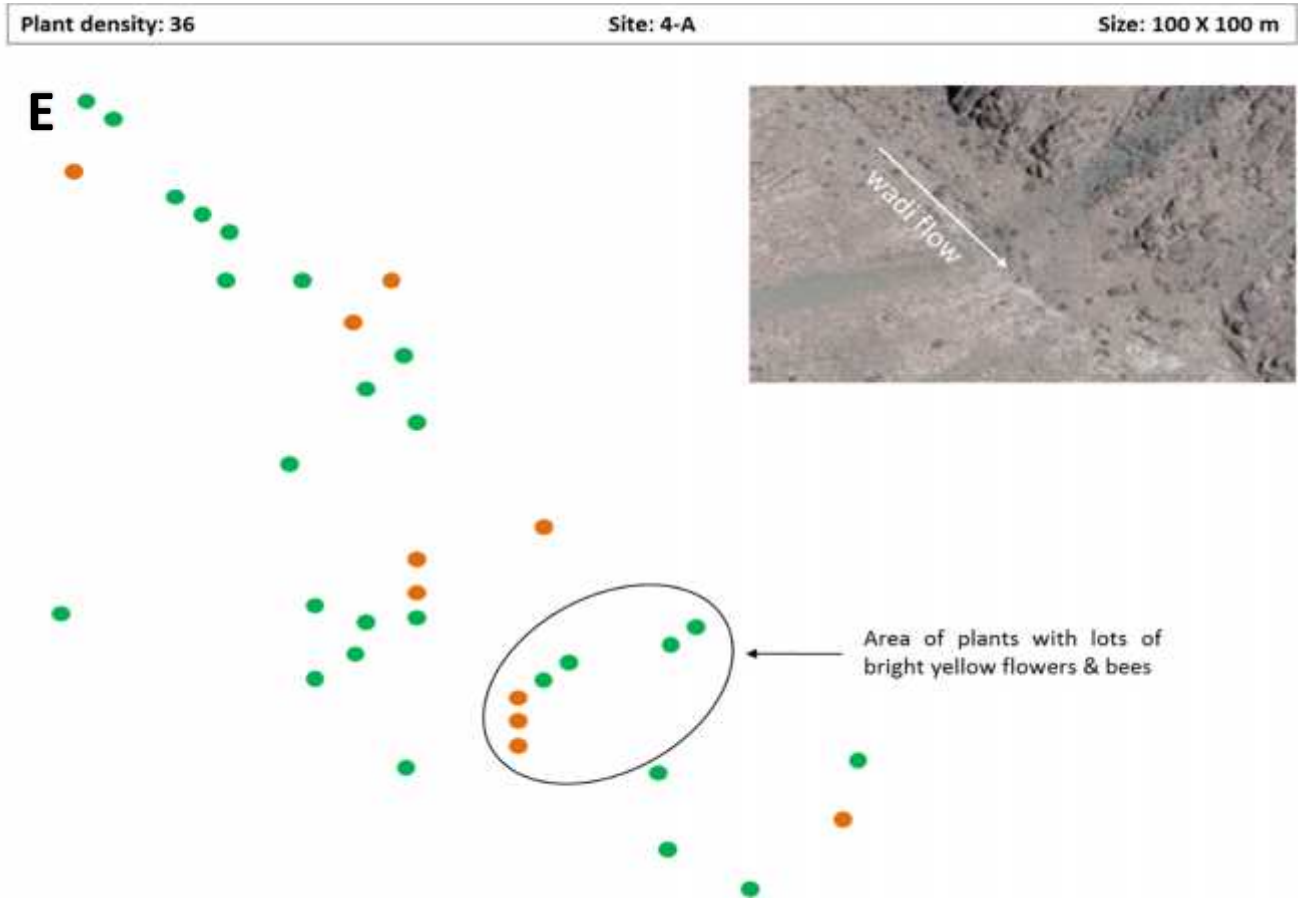


Figure S1: Map of Alkanna orientalis at each study site (a-e) on the eastern flank of Jabal El-Loz (Tabuk region, NW Saudi Arabia). The circles are the positions of Alkanna orientalis plants in the 100 x 10 -m plots; the green circles were plants that were randomly chosen for observation.



Figure S2: Photographs of some taxa found on or visiting the flowers of *Alkanna orientalis* in Jabal El-Loz, Tabuk Province, NW Saudi Arabia.

Numbers in photos are column 1 in table 2.2.



Fig S3: Images of A and B is to show the differences between male (A) and female (B) of *Anthophora pauperata*; each has been marked with a numbered disc stuck to their thorax. Image of C showed the right foretarsus of male *Anthophora pauperata*. Image of D showed the galea of *Anthophora pauperata*.



Fig S4: Physical contact in *Anthophora pauperata*: (A) aggressive male-male interaction; (B) male-female interaction. All interactions ended by falling onto the ground.