"The desert bees (*Apis mellifera* L) of Libya"

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Thank God for all his blessings

My family
for continuous and unconditional support

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Summary

Apis mellifera is endemic to Africa, Europe and western Asia. Its biogeography was addressed based on morphomtery. There was a gap of the biogeography in North Africa. In this thesis honeybee populations of *A. mellifera* in Saharan and coastal locations in Libya were investigated, morphologically and using mitochondrial DNA, to fill this gap. It is proved that the Libyan honeybees are distinctly different from both the adjacent *A. m. intermissa* bee populations of western northern Africa and those of *A. m. lamarckii* of Egypt in respect of morphology and mtDNA haplotypes. But more similar morphologically to *A. m. sahariensis*, suggesting that those populations might be derived from a formerly extended Saharan honeybee population during the Holocene pluvial. In spite of large imports of *A. m. ligustica* these apparently had minor impact on the endemic Libyan honeybee populations. Moreover, a contact zone between the evolutionary lineages A and O was identified in northwestern Libya. It was proven that the honeybee population of the Saharan oasis Kufra is isolated from the other locations for thousands years. In this thesis I presented a tool kit of 18 microsatellite DNA markers comprising a set of six unlinked loci, and three sets of four tightly linked loci which can be run in two multiplex PCR reactions. It was proven to be most effective in determining the number of colonies in a honeybee population, the parentage of workers in a colony and the mother genotypes of drones sampled in the wild.

Zusammenfassung

Die westliche Honigbiene *Apis mellifera* L. kommt in Afrika, Europa und im westlichen Asien vor. Die geographische Verbreitung der Unterarten wurde mit Hilfe morphometrischer und genetischer Methoden aufgeklärt. Bislang war die nordafrikanische Region nicht näher analysiert worden. Diese Lücke in der Biogeographie der Honigbiene sollte im Rahmen dieser Doktorarbeit geschlossen werden. Dazu wurden Honigbienen-Populationen in der Sahara und den küstennahen Regionen in Libyen sowohl mit morphometrischen als auch mit molekularbiologischen Methoden untersucht. Libysche Honigbienen-Proben unterscheiden sich sowohl morphologisch wie auch in der Sequenz der mitochondrialen DNA distinkt von den benachbarten Unteraten *A.m. intermissa* aus Nordwest-Afrika und der ägyptischen Unterart *A.m. lamarckii*. Sie sind morphologisch am nächsten mit *A.m. saharensis* verwandt. Es ist wahrscheinlich, dass die rezenten libyschen Honigbienen von Honigbienen-Populationen abstammen, die in der relativ niederschlagsreichen, d.h. pluvialen Phase des Holozäns in der Sahara weit verbreitet waren. Dagegen haben sich die massiven Importe der italienischen Honigbiene *A.m. ligustica* nach Libyen nicht im großen Ausmaß und in dieser Arbeit nicht messbar auf die Populationen der endemischen libyschen Honigbiene ausgewirkt. Darüberhinaus konnte eine Kontaktzone zwischen den mitochondrialen Abstammungslinien A und O in der nordwestlichen Küstenregion Libyens identifiziert werden. In der Oase Kufra, die in der Sahara im Südosten Libyens liegt, wurde eine Honigbienen-Population mit bislang unbekannter mitochondrialer Sequenz entdeckt, die sich offenbar seit mehr als tausend Jahren isoliert von den anderen Honigbienen-Populationen entwickelt hat. Schließlich wurde im Rahmen dieser Arbeit eine Methode entwickelt, die sich als effektivste in der Bestimmung von Kolonie-Zahlen in einer Honigbienen-Population, in der Analyse der Eltern von Arbeiterinnen in einem Bienenvolk und der Entschlüsselung der mütterlichen Genotypen von wild gefangenen Drohnen erwies. Sie basiert auf 18 Mikrosatelliten-DNA-Markern, die aus Primern für sechs nichtgekoppelte Loci und drei Primerpaaren für vier nahe gekoppelte Loci besteht, die in lediglich zwei Multiplex-Reaktionen analysiert werden können.

Keywords: *Apis mellifera*, North Africa, Libya, oases, morphomtery, mtDNA, microsatellite, conservation, contact zone, isolation

1. Introduction

1.1 Biogeography of *Apis mellifera*

Apis mellifera is endemic to Africa, Europe and parts of western Asia (Figure 1.1) ranging from Kirgisia in the east to the most western limits of Europe; from southern tip of Africa to the northern limits in Europe in south Scandinavia (Ruttner 1988; Sheppard and Meixner 2003). In this huge distribution range, *A. mellifera* can be found in a vast range of habitats ranging from desert to rain forests and from mountainous regions to plains (Smith 1961).

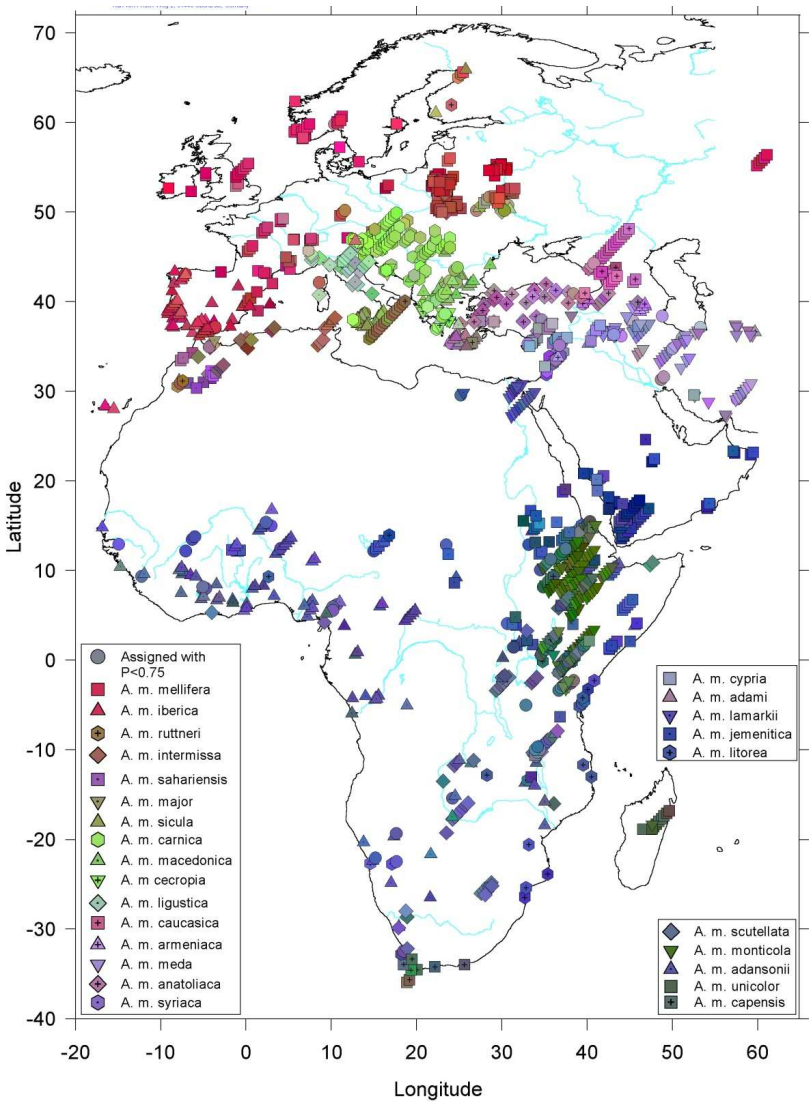

Figure 1.1. The subspecies distribution of Honeybees (*Apis mellifera L.*) (Fuchs 1998)

Because of this variety of habitats, climatic conditions, and floras as well as separations factors, it is not surprising that *A. mellifera* has split into numerous subspecies (races) about 0.3-1.3 myr ago (Ruttner 1988; Cornuet and Garnery 1991b; Arias and Sheppard 1996). Around 29 subspecies are currently recognized based on morphometric analyses (Ruttner 1988; Engel 1999; Sheppard and Meixner 2003). Each race is characterized with a set of distinctive characteristics probably as a result of local adaptation to the various regions (Louveaux 1966).

Ruttner et al. (1978) hypothesized that *A. mellifera* radiated from the Near East into in three different branches; (A) the branch which distributed in South and Central Africa, (M) the branch of Western Europe and North Africa, and (C) the branch of North Mediterranean. Ruttner himself (1988) added a fourth branch that includes near and Middle Eastern subspecies, naming it as the (O) lineage. These four lineages were, in principle, confirmed using mitochondrial DNA (mtDNA) and restriction fragment length polymorphisms (RFLP) but the subspecies of Northwestern Africa and *A. m. iberica* were assigned to branch A instead of M and *A. m. lamarckii* and *A. m*. *syriaca* to branch O (Garnery et al. 1993; Franck et al. 2000b, 2001). In addition, a fifth branch, termed Y has been recently described by Franck et al. (2001) comprising the subspecies of *A. m. jemenitica*.

1.2 Spread of honeybees by man

Apiculture is an important part of human culture and the relationship between humankind and honeybees is probably as old as man himself. Prehistoric cave paintings indicate that the interest of humankind for honey already existed in the Paleolithic period. About 4000 years ago, Egyptians used clay pots to keep bees for honey production but also to harvest other bee products including propolis and wax (Crane 1999).

Man developed techniques and equipments to facilitate the management of honeybees finally resulting in modern apiculture, which is based on removable combs and a hive systems allowing for easy honey production and colony transport. Long distance transport allowed the European colonists to carry the honeybees to the new world, where no natural occurrence of *A. mellifera*. Both European and African races have been introduced in the last few hundred years to America. For example, in 1622, Black German honeybees were exported from England to Virginia in North America, later in 1630 and 1633, other shipments arrived to Massachusetts. Then, through natural swarming, and migratory beekeeping, the honeybees

spread in the whole continent. In the period between 1788 and 1898, the spreading of *A. mellifera* around the globe was completed when English colonists carried bees to Australia, New Zealand and Tasmania (Crane 1999).

In an effort to improve beekeeping productivity, presumably superior commercial lines of *A. m. carnica* and *A. m. ligustica* have been introduced worldwide into apiculture (Franck et al. 2000a; Sušnik et al. 2004). In some countries strict breeding programmes aimed at replacing local populations by introducing foreign subspecies (Kauhausen-Keller and Keller 1994) or, at least, changes in the genetic content of those races. Nevertheless, the racial lines of European origin have generally been maintained in spite of these apicultural activities (Franck et al. 1998; De La Rúa et al. 2001, 2002, 2003). Striking disasters that resulted from man introduced honeybees are well documented in South America (Africanized bees) and South Africa (The capensis calamity).

1.2.1 The Africanized bee problem

European honeybee races originally introduced by European settlers were poorly adapted to tropical environments of South America and only poorly survived without intensive management. Therefore, Warwick Kerr had the brilliant plan to introduce African honeybees *A. mellifera scutellata* from South Africa into Brazil in 1956 (Nogueira Neto 1964; Kerr 1967). After introduction, an accidental release of some queens (Spivak et al. 1991) and a broadly planned breedaing concept caused the spread of *A. m. scutellata* throughout South and Central America. (Roubik and Boreham 1990; Winston 1992) and they reached the United States in 1990 (Hunter et al. 1993). Because of the African origin of these bees and to differentiate them from the original honeybees in Africa, they are called "*Africanized*" honeybees (Pinto et al. 2004). The successful invasion of the Africanized honeybees may resulted from the high adaptability to tropical ecological conditions (Diniz et al. 2003), swarming behavior (Winston et al. 1981) shorter generation time and smaller colonies (Hepburn and Radloff 1998). The introduction of Varroa to South America in 1970´s (Alves *et al.* 1975; Orosi-Pal 1975; Grobov 1976; Montiel and Piola 1976) and to USA in 1987 caused further losses in European populations (managed and feral) (Kraus and Page 1995; Pinto et al. 2004) and enhanced the spread of the Africanized type because they less susceptible to the those mites than the European honeybee colonies.

Box 1: The specific mating biology of *Apis mellifera*

All species of the genus Apis show high levels of polyandry (Oldroyd et al. 1998). The degree of polyandry also varies among the different subspecies of *A. mellifera*; however, *A. mellifera* queens can mate up to 45 drones (Moritz et al. 1995; Neumann and Moritz 2000). The mating system of honeybees is characterized by ‘‘drone congregation areas’’ (DCA’s) where the drones, from many colonies assemble (Estoup et al. 1994). Virgin queens of 1-2 weeks old make one or more ‘‘nuptial flights’’ and fly many kilometers to visit one or more DCA (Woyke 1964), and mate with many drones on a single flight (Ruttner 1988). The drone mates once and dies after mating. The queen stores the semen in the spermatheca, which will be used to fertilize the eggs throughout her lifetime. Once the queen starts oviposition, she never mates again (Winston 1987). After mating, the queen returns to the colony with semen load from numerous drones stored in the spermatheca. Unequal male contributions result from the polyandry and cause a diverse family composition in the colony (Estoup et al. 1994). The queen can either produce females by allowing sperms flow to fertilize eggs which develop into workers or queens depending on the nutrition of the young larvae or produce male by laying unfertilized haploid eggs.

1.2.2 The capensis calamity

Migratory beekeeping, where beekeepers follow rewarding honey flows is also an important technique in commercial beekeeping operation. However, long distance colony movements can cause problems whenever non-native subspecies are transferred into the range of other subspecies. The phenomenon "capensis calamity" is an ill famed example for the effect of migratory beekeeping on wild and managed honeybee populations. In 1990, migratory beekeepers transported thousands of honeybee colonies of *A. m. capensis* from the Cape region into the northern regions of South Africa, which resulted in the destruction of hundred-thousands of *A. m. scutellata* colonies (Neumann and Moritz 2002). The mechanism of the spreading of *A. m. capensis* into the territory of *A. m. scutellata* arose from the ability of capensis workers to produce female offspring (thelytoky, Lattorff et al. 2005); as well, they establish themselves as pseudoqueens. The pseudoqueens lay viable eggs and produce queen-like pheromones, they can be social parasites when enter a foreign colony and producing (thelytokousley) parasitic workers. Usually, the queens of *A. m. scutellata* can kill the capensis workers unless they are not much. In the later situation the queen loses the war. Then, wining capensis worker undertakes and uses the *A. m. scutellata* colony to breed its worker offspring, which will lead, in the end to the collapse of *A. m. Scutellata* colony (Neumann and Hepburn 2002).

1.3 Beekeeping practice and conservation

Apiculture has a major impact for the conservation of the endemic honeybee-races in the old world (Ruttner 1969; Ruttner 1988; Moritz et al 2002). The mating system of honeybees is hard to control, so that gene flow between the native and introduced honeybee subspecies is common (Sheppard et al. 1991a,b; Franck et al. 1998) and introgression can proceed very fast. In addition, beekeepers often move their apiaries from area to another either following nectar flows or for pollination purpose. Hence, autochthonous races are at risk to be replaced by commercial lines of preferred races such as *A. m. carnica* and *A. m. ligustica* to increase the honey yield. Furthermore, the parasitic mite *Varroa destructor* has spread worldwide due to the commercial transport of honeybees and migratory activities of beekeepers (Sumpter and Martin 2004) causing dramatic losses of honeybees around the world and requiring constant chemical treatments of the colonies to survive.

The conservation of local races of *A. mellifera* was proposed by (Ruttner et al. 1990, from Strange et al. 2007) in response to tangible hybridization of those population with imported stock. Several conservation programmes have been established to conserve the autochthonous races in their original ranges of distribution (Nikolenko and Poskryakov 2002; Sušnik 2004; Jensen et al. 2005a; Strange et al. 2007). The most important requirement for the conservation programmes to be successful is preventing hybridization with other subspecies (Jensen et al. 2005a). Therefore, the genetic integrity of the isolated populations (e.g., islands, oases, the Nile river valley) by the introduced races may less endangered than the others that are not isolated (Ruttner et al. 1978; Ruttner 1988; Sheppard et al. 1997).

Conserving the native races in their original range of distribution has many advantages, in addition to the ethical value of conservation, the adaptation strategies of endemic races to the local conditions, their resistance against the local diseases and parasites may prove to be important also from an apicultural point of view.

The historical background of a population is important to understand its role for conservation genetics. It is essential to assess the genetic isolation of threatened populations (Franklin and Frankham 1998) to prevent hybridizations with introduced stock. Only if we develop sustainable strategies to prevent hybridization, we can protect these populations as lasting genetic resources of the species.

1.4 Honeybees in Northern Africa and desertification

North Africa experienced consecutive cycles of aridity and moistness. About, 150,000 years ago the conditions were generally drier than those today with extended deserts spanning throughout North Africa. (Andel and Tzedakis 1996). Later, during the Eemian Interglacial (~125,000-120,000 y.a.) the North African climate was characterized by high rainfall (Frenzel et al. 1992; Andel and Tzedakis 1996). Following the Eemian interglacial (110,000 - 90,000 y.a.) desert conditions existed in some parts of West and South Africa while strong aridity occurred in other parts of the continent (Stokes et al. 1997). The climate became more variable across North Africa during the period between 110,000 and 11,000 y.a. The cold ice-rafting phases in the North Atlantic (Heinrich events) are thought to correspond with the most intensely dry and cool phases across Northern Africa and Arabia. The divergences between honeybee subspecies from northern and southern sides of the Sahara may have occurred during the late Pleistocene (~ 15 000 years BP) when the Sahelian zone became a desert while the northwest of Africa characterized by Mediterranean-like vegetation with most favourable conditions for honeybees. About ten thousands years ago, the conditions in north and central Africa became less arid and were much moister than at present. During that period, the Sahara desert disappeared (Lezine 1989; Ritchie 1994) which allowed for a population expansion and possible gene flow between the honeybees of North Africa and the Sahel. About 7,500 years ago aridity returned (Gasse and van Campo 1994; Alley et al. 1997) and the conditions across North-, Central- and East-Africa became much drier than before culminating in an arid phase about 3,800 y.a. (Petit-Maire and Gua 1996). Since then, the region was characterized by huge deserts creating today subspecies: *A. m. intermissa* along Mediterranean coast from Morocco through Algeria (Barour et al. 2005) to Tunisia (Lebdi-Grissa 1991a,b), *A. m. sahariensis* in the Saharan oases and the valleys along the northern edge of Sahara south of the Atlas mountain ridge (Hepburn and Radloff 1996), and *A. m. lamarckii* along the Nile Valley in Egypt (Ruttner 1988).

1.5 The tools to study honeybee biogeography and evolution

Biogeography, biodiversity, taxonomy and evolutionary history of honeybees *A. mellifera* was addressed on basis of morphometrics (Ruttner et al. 1978), allozymes (Nunamaker and Wilson 1981b; Sylvester 1982; Sheppard et al. 1991b; Smith and Glenn 1995), mitochondrial DNA (Cornuet and Garnery 1991b; Moritz et al. 1994) and nuclear DNA (Franck et al. 1998; De la Rúa et al. 2002).

1.5.1 Morphometric classification

Morphometric analysis of *A. mellifera* was first used by Cochlov in 1916 (Ruttner 1988), in search for bees with a long proboscis for the effective pollination of red clover. However, the early attempts to classify honeybees were based on individual morphometric characters without statistical analysis based on size and colour. Alpatov and Goetze introduced biometrics (Alpatov 1929; Goetze 1940) and in addition to the tongue length, Alpatov included more characters such as femur, tibia, metatarsus, length and width of wing and size of wax mirror. Goetze (1940, 1964) introduced more quantitative taxonomic characters to Alpatov‘s list, including indices of venation pattern of the forewing and length of hairs of the abdominal tergites, which were highly efficient in discriminating among European races. Louis (1963) also made an extensive study on the geographical variability on crossing points of wing veins. In subsequent studies Louis et al. (1968) could discriminate honeybee races, ecotypes within a race and even genetic lines with multivariate morphological analyses.

The recent classification of honeybees is based on numerical taxonomy and multivariate statistical-analyses initially promoted by DuPraw (1964, 1965) who was the first to use discriminant analysis on characters of the wing venation pattern. Subsequently, this analysis was further developed by Ruttner (Ruttner et al. 1978; Ruttner 1988) and Daly (Daly 1991, 1992). Ruttner et al. (1978) established a standard set of thirty-sex characters to classify the honeybees of the world. Daly and Balling (1978) could distinguish between Africanized and European honeybees in South America using quantitative characters of wing venation.

Principal Component Analysis and Factor Analysis were used to identify morphoclusters of colonies within populations (Ruttner et al. 1978; Ruttner 1988). Step-wise discriminate analysis is used to determine the most discriminatory variables and to calculate the percentage of correctly classified colonies. It allowed optimizing the number of selected characters, based on the region under investigation (Daly and Balling 1978; Ruttner et al. 1978). Dendrograms and Mahalanobis distances were introduced to draw the distances between clusters (Tomassone and Fresanaye 1971; Cornuet et al. 1975; Cornuet and Garnery 1991a, b; Daly 1992). With the rise of digital optical equipment and computers, the time of measuring and data analyses could be dramatically reduced (Daly et al. 1982; Meixner 1994) and finally upgraded to a fully automated system (Steinhage et al. 1997, 2001).

1.5.2 Allozymes

Morphometric analysis has limitations when it comes to measurements of genetic diversity within populations. Therefore, it was a major methodological breakthrough when it became possible to use allozyme variation, (allelic variants of enzymes controlled by a single locus) to assess

population differentiation. Tripathi and Dixon (1968) were the first to use the technique in honeybees and observed a marked difference of non-specific esterases (EST) patterns in the hemolymph of queen and worker larvae of honeybees *A. mellifera*. In a subsequent study, they also reported on caste specific differences in the number of malate dehydrogenase (MDH) isozymes in *A. mellifera*; they found two and three MDH isoenzymes in the hemolymph of queen and workers, respectively. Seven allozymes are known to be polymorphic, out of more than forty investigated enzymes in honeybees. Of these, cytoplasmic malate dehydrogenase (MDH) has proven to be the most useful one to study honeybee populations (Sylvester 1976; Cornuet 1979; Lobo et al. 1989; Cornuet and Garnery 1991a). This tool became particularly popular in conjunction with the Africanized honeybee problem. Nunamaker and Wilson (1981b) and Nunamaker et al. (1984) suggested that the MDH isozymes could be a diagnostic tool to identify the African honeybee *A. m. scutellata*. Most isozyme studies, therefore, are concerned with the Africanized bees in South and Central America (Nunamaker and Wilson 1981a; Sylvester 1982; Nunamarker et al. 1984; Del Lama et al. 1988, 1990; Sheppard et al. 1991b), comparing honeybees in America with those from South Africa and Europe. Subsequently, allozyme polymorphisms were used to study honeybee racial relationships and population structure in Africa (Ndiritu et al. 1986; Sheppard and Huettel 1988; Meixner et al. 1994); Europe and the Mediterranean basin (Cornuet 1982; Badino et al. 1983, 1984; Sheppard and Berlocher 1984, 1985; Sheppard and Mcpheron 1986; Cornuet and Garnery 1991a; Smith et al. 1991; Smith and Glenn 1995). Although the success of allozymes as a tool is not susceptible to environmental effects, they exhibit a relatively low level of polymorphism in honeybees (Hepburn and Radloff 1998). This can be as the consequence of the haplodiploidy system in the species (Pamilo and Crozier 1981).

1.5.3 mtDNA

The development to the in vitro amplification of DNA with the polymerase chain reaction (PCR, Mullis 1990) allowed for the direct use of DNA samples (nuclear and mitochondrial) as a powerful tool for the study of populations. DNA analyses overcome the limitations of morphometry and the low levels of polymorphism of allozymes for population genetic analyses. Mitochondrial DNA is the circular DNA

located in mitochondria, which are organelles in the cytoplasm of the cell. Each mitochondrion contains about two to ten mtDNA copies (Wiesner et al. 1992). In most animals, mtDNA encodes 13 proteins, two ribosomal RNAs, and 22 tRNAs, it has a region controlling replication and is typically devoid of other known functions. The size of mtDNA ranges between 14 kb and 42 kb. Nearly all of the mtDNA in a fertilized egg originates from the mother only, in contrast to nuclear DNA, which is inherited from both parents. Because of the

lack of recombination, the maternal transmission and the high mutation rate of animal mtDNA (Brown et al. 1979), mtDNA polymorphisms are powerful tools in phylogenetics and population genetics.

The mtDNA of various insects including several *Drosophila species,* the mosquitoes, locusts and also the honeybee *A. mellifera*, have been completely sequenced (De Bruijn 1983; HsuChen et al.1984a, b; Clary and Wolstenholme 1987; Satta et al*.* 1987; McCracken et al. 1987; Uhlenbusch et al. 1987; Vlasak et al. 1987; Garesse 1988). They all share the common pattern of a very high A+T content.

Most of the large-scale size variation lies in the control region of the molecule. In many organisms this is the so called D-loop, which is however lacking in the honeybee (Cornuet and Garnery 1991b). The mtDNA of *Apis mellifera* contains a second region, which is similar to the control region (Crozier and Crozier 1993)**.** It is located in the COI-COII intergenic region and contains various stretches of AT rich repeated sequences.

Moritz and Hawkins (1985) were the first to isolate the mitochondrial DNA of *Apis mellifera*. The isolated mtDNA digested with restriction enzymes, which showed a polymorphism in length and restriction sites (Moritz and Hawkins 1985, Moritz et al. 1986, Smith and Brown 1988). Later, Crozier and Crozier (1993) determined the sequence of mitochondrial DNA in *Apis mellifera*. The size of mtDNA of *A. mellifera* varies between 16.5 and 17 kb length depending on the subspecies (Smith and Brown 1988). This size variability is primarily based in the control region and $tRNA^{leu}$-cox2 (formerly COII) intergenic region (Smith and Brown 1990). The detailed sequence knowledge allowed for designing primers to use PCR for the selective amplification of specific mtDNA regions. For example the 16S (Bouga, 2005; Collet et al. 2007), cytochrome b (Crozier et al. 1991; Collins et al. 2000; 2006b), ND 5 gene (Bouga 2005) and the region which includes the tRNALeu gene, the $tRNA^{leu}$-cox2 intergenic region and the 5' end of the cox2 gene (De La Rúa et al. 1998; Franck et al. 2000a, b; Segura 2000; Diniz et al. 2003; Collet et al. 2006; Kandemir 2006a; Il'yasov 2007; Kozmus et al. 2007; Miguel 2007; Suppasat et al. 2007). MtDNA polymorphisms were used to discriminate among honeybee subspecies (Cornuet and Garnery 1991b, Crozier et al. 1991, Hall and Smith 1991, Garnery et al. 1993, Moritz et al. 1994, Garnery et al. 1995). The tool was also used to study the patterns of gene flow between introduced European and African honeybees in the New World (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991a; Moritz and Meusel 1992). In addition, because of the strict maternal inheritance, honeybees of hybrid origin do not carry a mixture of mtDNA's, they show only the pattern of their queen (Cornuet and Garnery 1991a, b; Smith 1991; Meusel and Moritz 1993) making the molecule particularly powerful in the study of hybrid zones (Smith et al. 1989; Moritz et al. 1994) and is an excellent tool for studying colonization processes (Garnery et al. 1992).

The tRNA^{leu}-cox2 intergenic region has attracted special attention because of its restriction site length polymorphisms (RFLP) (Crozier et al. 1989; Garnery et al. 1992, 1993; Moritz et al. 1994; Garnery et al. 1995; Franck et al. 1998, 2000b, 2001). The RFLPs of the tRNA^{leu}-cox2 intergenic region result from the variability of a specific P and Q sequence which can be best revealed by endonuclease *Dra* I restrictions. *Dra* I RFLPs are powerful tools to discriminate among the various biogeographic lineages. There are four types of P segments Figure 1.2; P (54bp) which exists in lineage M, P0 (67-bp) in lineages A and O, P1 (52bp) in lineage A and P2 (49) in lineage Y. But C lineage does not include P region. Q region is up to four copies of 192- 196 bp. The examination of the available tRNA^{leu}-cox2 intergenic region sequences led to use the endonuclease *Dra* I, which has the recognition site is TTTAAA, should show a significant amount of polymorphism (Garnery et al. 1993).

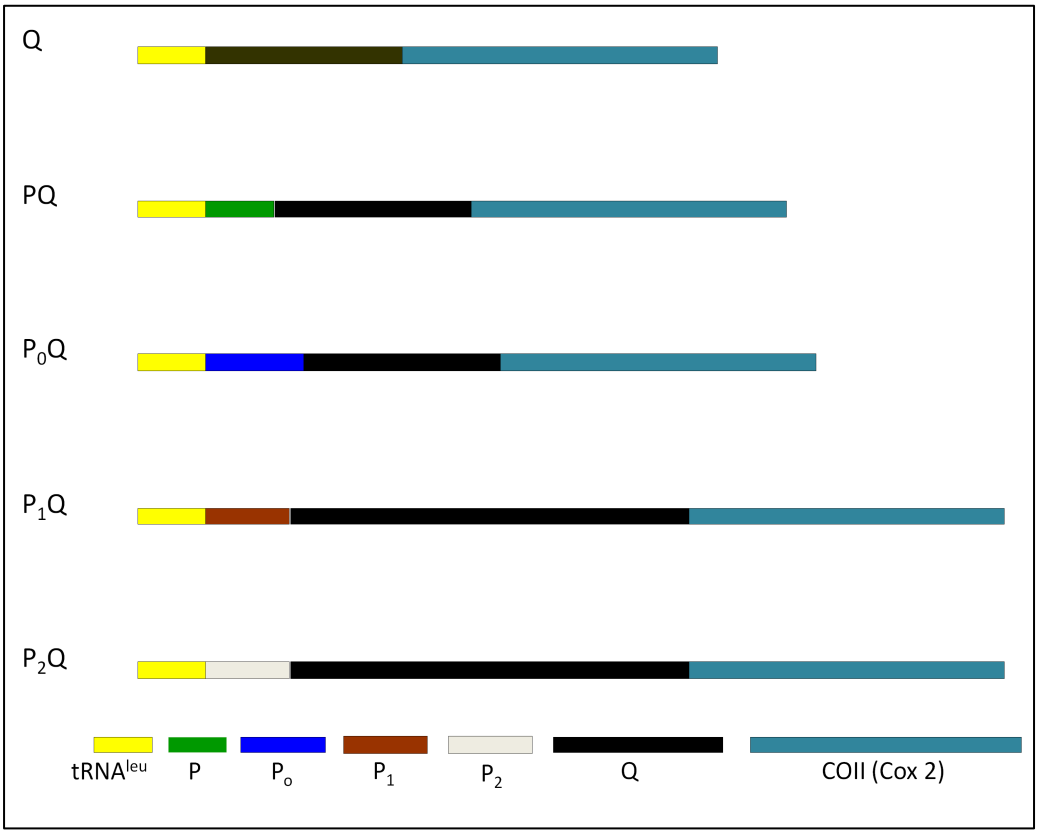

Figure 1.2. Various length polymorphisms of the tRNA^{leu}-cox2 intergenic region

1.5.4 Microsatellites

Microsatellite DNA, or Simple Sequence Repeats (SSRs), are highly polymorphic DNA markers that involve a variable number (up to 100) of tandem repeats of 1-6 nucleotides. They are present in both nuclear and organelle DNA and occur in high number in all prokaryotic and eukaryotic

genomes (Zane et al. 2002; Turnpenny and Ellard 2005*).* The high degree of polymorphism of microsatellite markers results from the high mutation rate in the genome regions *(*Jarne and Lagoda 1996*).* Today microsatellite DNA markers are used in wide scale and have given rise to the discipline of molecular ecology, soon after their first description (Litt and Luty 1989; Tautz 1989; Weber and May 1989).

Microsatellite DNA markers are superior to mtDNA markers for fine scale population analyses, because they are more variable than the mitochondrial markers. Therefore, they are preferentially used for paternity testing, population differentiation, population structuring, linkage analysis, genetic mapping and ancient and forensic DNA studies (e.g. Hazan et al. 1992; Serikawa et al. 1992; Sirugo et al. 1992; Jarne and Lagoda 1996; Schuler et al. 1996; Knapik et al. 1998; Jensen et al. 2005a). A pair of specific primers is used to amplify a certain microsatellite locus in process of PCR and the obtained fragments can be easily identified by their length polymorphism.

Although microsatellites are excellent markers, also they may suffer from unexpected pitfalls. For example, the technical problem of 'null alleles' that can result from point mutation in the primer annealing sites, and microsatellites fail to amplify in PCR assays can cause interpretation problems (Jarne and *Lagoda* 1996*;* Dakin and Avise 2004). Another problem can arise from 'homoplasy' which are alleles similar in length, but of different descent. They can be identical in both length and sequence or only identical in length but different in sequence. Since the size detection is the most used way to identify the locus, size homoplasy is a notorious problem in interpreting genotypes that may lead to misidentification (Yokoyama et al. 2004).

Microsatellites developed for particular species can often be used in closely related species, but typically the percentage of loci that successfully amplify decreases by increasing phyologenetic distance.

In *A. mellifera*, several hundreds of microsatellite markers had been characterized (Rowe et al. 1997; Solignac et al. 2003) well before the sequencing of the full genome (Weinstock et al. 2006). Microsatellites have been used to address questions concerning the origin of species and subspecies of honeybees (Franck et al. 1998), to assess the number of patrilines in a honeybee colony and fitness (Estoup et al. 1994; Kraus et al. 2003), to determine mating range and polyandry (Moritz et al. 1995; Jensen et al. 2005b) to study genetic structure of population (De la Rúa et al. 2002, 2004; Sušnik et al. 2004; Kraus et al. 2005; De la Rúa et al. 2006; Bodur et al. 2007), and mapping studies (Weinstock et al 2006; Lattorff et al. 2007).

Recently the use of tightly linked microsatellite loci has been shown to be particularly informative to determine the number of colonies in a honeybee population (Moritz et al. 2008).

1.6 Aims of the work

In this Thesis, I will investigate the biogeography of *A. mellifera* in Northern Africa to unravel the transition from the oriental to the occidental subspecies. Moreover I will assess the impact of apiculture and transhumance on the biodiversity of endemic honeybee populations. I will hence study honeybee populations in remote Saharan oases with and without beekeeping and compare these populations with those of coastal regions in Libya with both morphometrical and molecular tools. This will fill knowledge gap in North Africa gap on honeybee biogeography and the subspecies distribution. The study will also clarify the transition from the African (A) to the Near East (O) evolutionary lineage, which is supposed to be some where in North Africa. Moreover, I want to:

1) evaluate the genetic structure of Libyan honeybee populations,
2) determine the differentiation between the investigated populations,
3) asses the impact of migratory beekeeping on those populations,
4) evaluate the effect of isolation, which resulted from the desertification,
5) address whether the honeybees in Libyan oases represent indigenous ecotypes, as old relic populations from a bigger population inhabited the region thousands of years ago, or have been introduced by man and are established by apiculture.

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2. Morphological study of Honeybees (*Apis mellifera* L) from Libya

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Abstract

We show, with classical morphometrical analyses, that Libyan bees sampled at coastal and desert locations are distinctly different from both the adjacent *A. m. intermissa* bee populations of Tunisia and Algeria and those of *A. m. lamarckii* of Egypt. The morphotype was most closely related to *A. m. sahariensis* and, based on wing venation angles, showed affinities to *A. m. jemenitica*, indicating that the sampled populations might be derived from a formerly extended Saharan honeybee population during the Holocene pluvial. Scattered morphometric similarities to the European bee *A. m. ligustica* suggest that importation of honeybees from Italy may have had only minor impact on endemic Libyan honeybee populations. Conservation measures might be particularly appropriate for remote oasis populations, which might be true relic population from the Holocene.

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3. *Apis mellifera* evolutionary lineages in Northern Africa: Libya, where Orient meets Occident

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Abstract

The distribution of various evolutionary lineages of *Apis mellifera* subspecies in Africa is still controversial. We sampled honeybees from eight coastal locations and three Saharan oases in Libya and analyzed mtDNA variability with restriction fragment length polymorphisms (RFLP) and the sequence of the tRNAleu-cox2 intergenic region. Haplotypes belonging to the oriental O evolutionary lineage, including four which are newly described, were detected in all investigated locations. Haplotypes belonging to the European M lineage were rarely detected, probably reflecting the effect of sporadic importations. Honeybees belonging to the A lineage were detected in Al Aziziyah and Zlitan close to the Tunisian border. The distribution of the O lineage extends westward up to the border between Libya and Tunisia, a contact area between the O and A lineages. Various Libyan honeybee populations in Saharan oases are characterized by novel and unique haplotypes (O4, O5, O5´ and O5´´). These might be natural relic populations that became isolated when the North African Sahara desert was still grassland (0.126 - 0.168 Myr ago).

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4. A microsatellite DNA toolkit for studying population structure in *Apis mellifera*

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Abstract

We present a set of 18 microsatellite DNA markers that can be run in two multiplex PCRs as standard tool for assessing molecular ecological problems in honeybees (*Apis mellifera*). In addition to a set of six unlinked loci testing for classical population genetic parameters, we present three sets of four tightly linked loci, each located on three different chromosomes. These linked markers are useful for determining the number of colonies in a population as well as the parentage of drones and workers. Moreover, the tool kit can test for various modes of natural selection in honeybee populations.

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5. 10000 years in isolation? Honeybees (*Apis mellifera* L) in Saharan Oases

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Abstract

After the transition from a savannah to a desert about 10000 years ago the isolated Saharan oases offer a unique case for studying the effect of population fragmentation and isolation over a period of many thousand years. We use the honeybee, *Apis mellifera*, as a test system because they are an abundant wild species in the African dry savannahs but are particularly sensitive to drift and bottlenecks in small isolated populations due to the small effective size resulting from male haploidy, the sex determination system and sociality. We compared the non-fragmented coastal population with the oases of Brak and Kufra using 15 polymorphic microsatellite loci assessing the mating frequency, colony density, gene diversity, and population differentiation. We found that the honeybee population of the remote oasis of Kufra is well isolated whereas those of the oasis of Brak and the coastal regions show genetic foot prints of introgression by commercial beekeeping. The isolated Kufra population showed no indications of inbreeding suggesting that the endemic population size is sufficient to ensure sustainable local survival.

Molecular Ecology in review (MEC-09-0540)

6. General Discussion

In this Thesis, I investigated honeybee populations of *A. mellifera* in Saharan and coastal locations in Libya to fill the North Africa gap of biogeography and distribution of honeybees, to clarify the transition from the African (A) to the Near East (O) evolutionary lineage, which is supposed to be some where in North Africa. Moreover, to asses the impact of migratory beekeeping on those populations, to evaluate the effect of isolation, which resulted from the desertification and to address whether the honeybees in Libyan oases represent indigenous ecotypes, as old relic populations from a bigger population inhabited the region thousands of years ago, or have been introduced by man and are established by apiculture.

6.1 The subspecies south of the Mediterranean

There are four morphometrically defined lineages of the honeybee (Ruttner et al. 1978; Ruttner 1988). All these branches (A, C, M and O) are found around the Mediterranean where they are represented by 15 identified subspecies: *A. m. sahariensis* and *A. m. intermissa* in northwestern Africa. (Cornuet et al. 1988; Ruttner 1988; Lebdigrissa et al. 1991a, b; Hepburn and Radloff 1998; Barour 2005). *A. m. lamarckii* in the Egyptian Nile valley (Ruttner 1988). *A. m. syriaca* and *A. m. meda* east of the Mediterranean (Ftayeh et al. 1994); *A. m. cypria* in Cyprus (Bouga et al. 2005; Kandemir et al. 2006); *A. m. anatoliaca* in Turkey (Ruttner 1988); *A. m. adami* in Crete (Ruttner 1980); *A. m. ruttneri* on the Island of Malta (Sheppard et al. 1997); *A. m. sicula* in Sicily; *A. m. cecropia*, *A. m. macedonica*, *A. m. carnica* and *A. m. ligustica* in north of Mediterranean; *A. m. mellifera* in France and *A. m. iberica* in the Iberian peninsula (Ruttner 1988).

Since the O lineage is represented by *A. m. lamarckii* in Egypt and the other North African subspecies *A. m. intermissa* and *sahariensis* in the west of the African Mediterranean belong to the lineage A, the contact zone of those lineages must to be somewhere between Egypt and Tunisia in North Africa. Thus, the morphometric analysis of the honeybees of Libya in this study provides an essential missing link to understand the distribution and spread of honeybees around the Mediterranean. As well to assess (morphometrially) the beekeeping practice on the investigated samples.

The investigated honeybee colonies sampled at coastal and desert locations in Libya showed that they are distinctly different from both the adjacent *A. m. intermissa* bee populations of western northern Africa and those of *A. m. lamarckii* of Egypt, but more similar to *A. m. sahariensis*. The venation angles analyses were similar to *A. m. jemenitica* suggesting that those populations might be derived from a formerly extended Saharan

honeybee population during the Holocene pluvial: a true relic population from the Holocene? In spite of large imports of *A. m. ligustica* these apparently had minor impact on the morphology of endemic Libyan honeybee populations.

6.2 The biogeography of honeybees around the Mediterranean based on mtDNA variability

Based on mitochondrial DNA (mtDNA), five evolutionary lineage were detected in old world (A, C, M, O and Y); four of them (A, C, M and O) are endemic around the Mediterranean Basin (Garnery et al. 1993; Arias and Sheppard 1996; Franck et al. 2000a, 2001; Miguel et al. 2007; Cánovas et al. 2008). The contact zones between these lineages have been detected based on molecular tools (Garnery et al. 1995; Smith and Glenn 1995; Franck et al. 1998, 2000a; De la Rúa et al. 2002; Kandemir et al. 2006; Dall´Olio et al. 2007; Miguel et al. 2007; Cánovas et al. 2008) except the contact zone of lineages A and O in northern Africa which is still unclear. So far, *A. m. lamarckii* of Egypt represents the western limit of lineage O (Arias and Sheppard 1996), while *A. m. intermissa* of Tunisia represents the most eastern distribution of this subspecies in North Africa (Hepburn and Radloff 1998). Therefore, analyzing honeybees sampled in Libya has a high interest to understand the biogeographic transition of lineages A and O in northern Africa. Moreover, mtDNA analyses will critically test whether honeybees in Libyan oases represent old relic populations and are indigenous ecotypes or have been introduced by man and are a result of apiculture.

Honeybees were sampled at eight coastal locations and three Saharan oases in Libya. The samples were analyzed for mtDNA variability with restriction length polymorphisms (RFLP) and the sequence of the tRNAleu-cox2 intergenic region.

Seven haplotypes belonging to three evolutionary lineages A, M and O were detected, four of which were newly described (O4, O5, O5´ and O5´´). In contrast to the morphometric analyses, there are indications for importations of commercial lines of European honeybees, since a haplotype (M3) was detected in two locations. The known distribution of the oriental lineage (O) extends westward and its contact zone with the African lineage (A) was detected near the border between Libya and Tunisia. Additional confirmation of the distinctness of Libyan honeybee populations in Saharan oases was obtained from characterizing novel and unique mtDNA haplotypes (O4, O5, O5´ and O5´´).

6.3 A microsatellite DNA toolkit for studying population structure in *Apis mellifera*

Since the genome sequence of *Apis mellifera* has been published (Weinstock *et al*. 2006) micorsatllite loci can be derived directly from the sequence offering more than 17 000 di- and 7000 trinucleotide loci (Benson 1999). This generates novel possibilities of using microsatellite loci. Whereas unlinked markers are preferable for classical population genetic problems, set of linked microsatellite loci are typically used for mapping studies in the honeybee genome (Lattorff *et al*. 2007). Moreover, tightly linked microsatellite markers are proven to be useful for determining the number of colonies in a honeybee population (Moritz *et al*. 2007a). In this thesis I presented a tool kit of 18 microsatellite DNA markers comprising a set of six unlinked loci, and three sets of four tightly linked loci. Each linkage group is located on three different chromosomes: 1) the first on chromosome 3 next to the sex locus (csd) which determines sex 2) the second set on chromosome 13 next to the thelytoky gene, which controls worker reproduction, 3) the third is on chromosome 16 in a large ”gene desert” expected to be selectively neutral. This tool kit was proven to be most effective in determining the number of colonies in a honeybee population, the parentage of workers in a colony and the mother genotypes of drones sampled in the wild. Moreover, it can be used to test for classical population genetic parameters. Because the analyses can be run in two multiplex PCR reactions it saves costs of time and money and is suggested as a standard tool for molecular ecological studies in honeybees

6.4 Isolation through desertification: the honeybees of Al Kufrah

The dramatic climatic changes of the Holocene Pluvial, lead to the desertification of the Sahara in North Africa. Nine thousand years ago the Sahara was a green savannah (Gasse et al. 1990; Hooghiemstra et al. 1992), a habitat to which *A. mellifera* is particularly well adapted. Today, the desertification confines the existence of honeybees to the oasis which might be old relic populations from a time when honeybees where abundant all over North Africa. The isolation of Kufra offers a unique opportunity to study a natural honeybee population under closed conditions, because even today the reaching the oasis by land is too difficult for migratory beekeepers.

As a consequence the honeybee, *Apis mellifera*, as an ideal test system to test impact of isolation through desertification. Moreover the species is particularly sensitive to drift and bottlenecks due to the small effective size resulting from male haploidy, the sex determination system and sociality. We compared the non-fragmented coastal population with the oases of Brak and Kufra using 15 polymorphic microsatellite loci assessing the mating frequency, colony density, gene diversity, and population differentiation. The

honeybee population of the remote oasis of Kufra was found to be well isolated whereas there are signs of introgression by commercial beekeeping in the populations of Brak and the coastal regions. Moreover, the isolated Kufra population showed no indications of inbreeding suggesting that the endemic population size is sufficient to ensure sustainable local survival.

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7. Appendix

7.1 Declaration on the Author Contributions

I. Shaibi T, Fuchs S, Moritz RFA (2009) Morphological study of Honeybees (*Apis mellifera*) from Libya. *Apidologie* **40**: 97-105.

I collected the samples, wrote the paper (65%). S Fuchs made the measurements, analyzed the data and participated in paper writing (30%). RFA Moritz supervised the work, revised the article and provided helpful discussions (5%).

II. Shaibi T, Muñoz I, Dall´Olio R, Lodesani M, De la Rúa P, Moritz RFA (2009) *Apis mellifera* evolutionary lineages in Northern Africa: Libya, where Orient meets Occident. *Insect Sociaux* **56**: 293-300.

I participated in samples collection, RFLP analysis, sequencing, data analysis and writing the paper (50%). I Muñoz participated in sequencing analysis and revision (25%). P De la Rúa participated in analysis and revision (10%). R Dall´Olio participated in sample collection (5%). M Lodesani participated in sample collection (5%). RFA Moritz supervised the work, provided helpful discussions and participated in revision (5%).

III. Shaibi T, Lattorff HMG, Moritz RFA (2008) A microsatellite toolkit for studying population structure in *Apis mellifera. Mol Ecol Resour* **8**: 1034–1036.

I collected Marzuq samples, genotyped the samples, choose the unlinked markers analyzed the data, made the primer optimization and wrote the paper (80%). HMG Lattorff designed primers of the linked marker sets, made the initial primer tests and participated in paper writing (15%). RFA Moritz supervised the work, revised the article and provided helpful discussions (5%).

IV. Shaibi T, Moritz RFA (in review) 10000 years in isolation? Honeybees *(Apis mellifera)* in Saharan Oases. *Mol Ecol*

I collected the samples, genotyped the samples, analyzed the data and wrote the paper (95%). RFA Moritz supervised the work, revised the article and provided helpful discussions (5%).

7.2 Acknowledgements

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Specials thanks to Prof. Dr. Hans Hinrich Kaatz for his valuable advices. Thanks to Petra Leibe, Beate Springer for their support during the lab work, to all my co-authors for their contributions and to the current and former members of the Halle lab.

Thanks to many Libyan beekeepers for their help in providing honeybees samples.

I am grateful to my father, mother, my wife and all members of my family for their support and love they gave to me. As well as, to my friends.

7.3 Curriculum vitae

Personal

Name	Taher Ahmed Khalifa Shaibi
Gender	male
Date and place of birth	20.05.1974, in Tripoli (Libya)
Nationality	Libyan
Martial status	married+2 children
Language	Arabic, English and German

Education

- 2005-2009: Ph.D student at the Martin-Luther-University, Halle-Wittenberg, Germany. Dissertation thesis “The desert bees (*Apis mellifera* L) of Libya” Supervised by Prof. Dr. Robin F.A. Moritz.
- 1998 – 2002: M.Sc in Zoology at Zoology Department, Science Faculty, AL-Fateh University Tripoli, Libya.
- 1992 – 1996: B.Sc in Zoology at Zoology Department, Science Faculty, AL-Fateh University Tripoli, Libya.

Employment History

- July 2002 - Now: Lecturer assistant at Zoology Department, Faculty of Science-Al-Fateh University/ Tripoli, Libya.
- July 2001 - June 2002: Teacher assistant at Zoology Department, Faculty of Science-Al-Fateh University/ Tripoli, Libya.

7.4 Publications

1. Shaibi T, Howege, HM (2004) Using of head width in aging of *Hemilepistus reaumuri* (Audouin, 1826) (Oniscidea: Porcellionidae) from Al-Khomes. *Lib J Basic Appl Sci* **13**: 91-113.

2. Shaibi T, Lattorff, HMG Moritz RFA (2008) A microsatellite toolkit for studying population structure in *Apis mellifera. Mol Ecol Resour* **8**: 1034–1036.

3. Shaibi T, Fuchs S, Moritz RFA (2009). Morphological study of Honeybees (*Apis mellifera*) from Libya. *Apidologie* **40**: 97-105.

4. Shaibi T, Muñoz I, Dall´Olio R, Lodesani M, De la Rúa P, Moritz RFA (2009) *Apis mellifera* evolutionary lineages in Northern Africa: Libya, where Orient meets Occident. Insect sociaux Online First (DOI: 10.1007/s00040-009-0023-3)

5. Shaibi T, Moritz RFA (in review) 10000 years in isolation? Honeybees *(Apis mellifera)* in Saharan Oases. *Mol Ecol.*

6. Jaffé R, Dietemann V, Allsopp MH, Costa C, Crewe RM, Dall'Olio R, De la Rúa P, El-Niweiri MAA, Fries I, Kezic N, Meusel MS, Paxton RJ, Shaibi T, Moritz RFA (in press) Filling the gap in pollinator decline censuses: Measuring the density of honeybee (*Apis mellifera*) colonies across their natural range. *Conserv Biol*

7.5 Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre, dass ich mich bisher noch nicht um den Doktorgrad beworben habe.

Ferner erkläre ich, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Halle (Saale), den 27. 05. 2009

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Taher Shaibi