Mitochondrial discrimination of honeybees (Apis mellifera) of Sudan

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Apidologie 39 (2008) 566–573
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DOI: 10.1051/apido:2008039

Available online at:
www.apidologie.org

Original article

Mitochondrial discrimination of honeybees (*Apis mellifera*) of Sudan*

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Received 13 December 2007 – Revised 15 May 2008 – Accepted 30 May 2008

Abstract – Sudanese honeybee populations are surrounded by a suite of various subspecies with different mitochondrial haplotypes, including the O-lineage in the north (Egypt), the Y-lineage in the east (Ethiopia) and the A-lineage in the south and west. Using *Dra* I analyses and the partial sequence of the tRNAleu COII region of 75 sampled colonies throughout Sudan, we never found the Y-lineage in Sudanese honeybees but instead seven different haplotypes from the A-, O-, and C-lineage (A_1, A_4, A_8, A_{13}, O_1', O_1 and C_2) suggesting that the Y-lineage is not common to Sudan. The mitochondrial haplotypes co-segregated with the highly diverse ecosystems in Sudan. Honeybees of the wet savannah and forest ecosystems showed the A-lineage, identical to *A. m. adansonii* and *A. m. scutellata*. The honeybees in the desert, semi desert, and dry savannah of Sudan have the O-lineage, similar to *A. m. lamarckii* and *A. m. syriaca*. Haplotype C_2 was found in apiaries with imported stock (*A. m. carnica*). This reclassification of the honeybees from Sudan has consequences for the interpretation of the biogeography of *A. mellifera* in the Maghreb and Mashriq regions.

***Apis mellifera jemenitica* / subspcies / mitochondrial DNA / Sudan / biogeography**

1. INTRODUCTION

The Western honeybee, *Apis mellifera* L., comprises a vast number of endemic subspecies in Europe, Asia and Africa. North and North-East Africa appears to be a region with a particularly high honeybee diversity (Whitfield et al., 2006) and five different subspecies of *Apis mellifera* have been taxonomically recognized from this area (Engel, 1999): *A. m. lamarckii* (Cockerell, 1906) in Egypt; *A. m. intermissa* (Maa, 1953) and *A. m. sahariensis* (Baldensperger, 1932) in Morocco; *A. m. litorea* (Smith, 1961) and *A. m. jemenitica* (Ruttner, 1975) in eastern Africa. However, the honeybees of Sudan, a region bridging many of these areas, have been neither intensively nor systematically sampled. The classification of the honeybees' native to Sudan has therefore been controversial. Although initially the subspecies included *A. m. nubica* Ruttner (1975), *A. m. sudanensis* (El-Sarrag et al., 1992) and *A. m. bandasii* (Mogga, 1988), today only a single subspecies is recognized, *A. m. jemenitica* (Ruttner, 1988; Engel, 1999), because the various taxa did not form discrete and separate morphoclusters (Hepburn and Radloff, 1998).

Mitochondrial DNA (mtDNA) has also been used to classify honeybee subspecies (Cornuet and Garnery, 1991; Smith, 1991; Garnery et al., 1992; Moritz et al., 1994). In particular, mtDNA variance in the COI-COII region has been extensively used to discriminate among the *A. mellifera* subspecies (Moritz et al., 1994, 1998; Garnery et al., 1995). Although the majority of African honeybees belong to a mitochondrial lineage termed "A" (Smith, 1991; Garnery et al., 1992, 1993; Arias and Sheppard, 1996., Franck et al., 2001) there is considerable mtDNA variability, particularly in the North East of the continent. The honeybees of North- and East Africa can

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be assigned to three different mtDNA lineages: A, O and Y. *A. m. intermissa* in Morocco and Algeria belong to the A lineage comprising eight different haplotypes (A_1–A_4, A_8–A_{10}, A_{13}). Five haplotypes of the A-lineage have been reported for the neighbouring subspecies *A. m. sahariensis* (A_1, A_3, A_4, A_8, and A_9) (Garnery et al., 1995). In Egypt *A. m. lamarckii* only carries the O-lineage haplotypes, whereas *A. m. jemenitica* from Ethiopia belongs to the Y-lineage detected by Franck et al. (2001). *A. m. litorea* from Somalia belongs to two different mtDNA lineages, O and A (Franck et al., 2001). As a result, Sudanese honeybee populations are surrounded by a suite of various lineages with the O-lineage in the north (Egypt) (Franck et al., 2001), the Y-lineage in the east (Ethiopia) and the A-lineage in the south and west (Franck et al., 2001). Given the vast size of the country and its highly diverse ecosystems spanning from deserts to tropical forests, one might expect considerable variability among the native honeybees of Sudan. If variable climatic conditions and ecosystems are important factors for natural selection shaping *A. mellifera* ecotypes and subspecies, it would be surprising to find only a single ecotype in the region. Since morphometrical analyses yielded no distinct morphoclusters (Hepburn and Radloff, 1998), we here focus on mtDNA variability of *A. mellifera* samples from Sudan as a tool for classification. We use the *Dra* I test of the COI-COII region in combination with sequence information to assign the biogeographic lineages of endemic honeybees. This data will also be an important base for developing coherent policies for the conservation of local honeybees in Sudan.

2. MATERIALS AND METHODS

2.1. Sampling

About 100 workers each from seventy five colonies were sampled from different localities in Sudan covering most diverse habitats, ranging from desert to tropical rainforest (Fig.1). Forty seven colonies were sampled in the wild and 28 colonies from three managed apiaries in Khartoum (2), Sinjah (6), and New Halfa (3) (Tab. I, Fig. 1). All sampled workers were preserved in 75% ethanol until

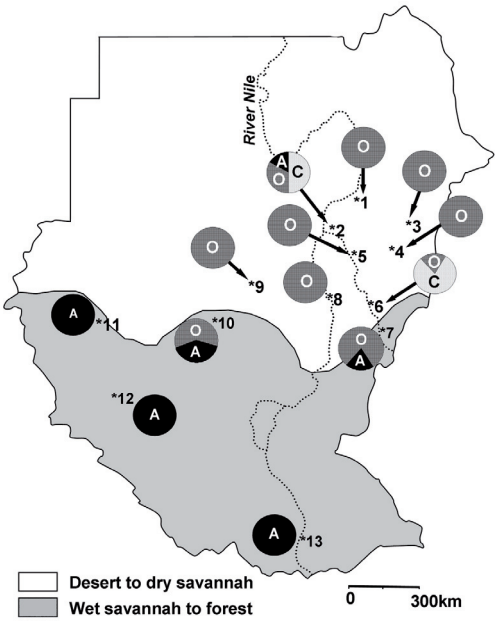

Figure 1. Vegetation zones of Sudan (El-Sarrag, 1977; Harrison and Jackson, 1958) and the distribution of COI-COII lineages at the sample locations (1 to 13). Pie charts indicate the frequencies of the mitochondrial lineages A, O, and C at each location. Managed colonies from apiaries at locations 2 and 6 had the non-native haplotype C. For more detailed haplotype information see Table I.

DNA extraction. The lower numbers of sampling locations (5) and sampled colonies (n = 18) in south and west Sudan compared to north and central Sudan (8 locations and 57 colonies) is due to severe sampling problems related to the Darfour war and the absence of any apiculture in these regions.

2.2. DNA extraction

Up to five worker bees per colony were subjected to mtDNA analysis. Workers were rinsed for two hours at room temperature and vacuum-dried overnight (Garnery et al., 1993). Total DNA was extracted from legs using the Chelex extraction method (Walsh et al., 1991).

2.3. PCR amplification and digestion

The COI-COII region of the mtDNA was amplified with standard PCR techniques in a total volume

Table I. Localities, coordinates, types and numbers of colonies, frequencies of haplotyps, ecological data and altitudes of sampling areas.

	Location	North	East	Type	n	A_1	A_4	A_8	A_{13}	O_1	O_1'	C_2	Habitat	Precipitation (mm/year)	Altitude
1	Shendi	16° 42'	33° 26'	wild	5					3	2		desert	25–74	360 m
2	Khartoum	15° 35'	32° 32'	apiary	8		1			3		4	semi desert	75–224	377 m
3	New Halfa	15° 20'	35° 35'	apiary	10					2	8		semi desert	75–224	459 m
4	Al Faw	14° 9'	34° 20'	wild	10					3	7		dry savannah	225–274	439 m
5	Wad Madani	14° 24'	33° 32'	wild	5					5			dry savannah	225–274	414 m
6	Sinjah	13° 9'	33° 56'	apiary	10						2	8	dry savannah	375–474	397 m
7	Dmazin	11° 46'	34° 21'	wild	4				1	1	2		wet savannah	475–724	487 m
8	Kosti	13° 10'	32° 40'	wild	5					5			dry savannah	225–274	380 m
9	Umm Ruwabah	12° 54'	31° 13'	wild	4					4			dry savannah	375–474	458 m
10	Nyala	12° 3'	24° 53'	wild	2	2							wet savannah	475–724	686 m
11	Hujaylij	11° 59'	27° 52'	wild	5		1	1		3			wet savannah	725–974	467 m
12	Raga	8° 28'	25° 41'	wild	3	3							wet savannah	725–974	603 m
13	Juba	4° 51'	31° 37'	wild	4	4							forest	975–1474	550 m

of 30 μL with 1× Taq buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of primers E2 and H2 (Garnery et al., 1992), and 1 U Taq-polymerase. The PCR program was as follows: 5 min initial denaturation at 96 °C, 30 cycles at 95 °C for 0.5 min, 1.5 min at 50 °C, and 1.5 min at 72 °C, with a final extension of 10 min at 72 °C. Five μL of the PCR product were electrophoresed in a 1.5% agarose gel for size determination, and 20 μL were treated with the restriction enzyme *Dra* I (0.5 U) at 37 °C for 4–12 h. Restriction fragments were separated on 8–10% acrylamide gels and stained with ethidium bromide.

2.4. DNA purification and sequencing

Haplotypes which were difficult to interpret based on restriction analyses alone were confirmed by sequencing both strands of the intergenic region including the noncoding region and the 5'-end of the COII gene (Franck et al., 2000a, 2001). PCR Purification of the amplified DNA fragments was achieved using the Pench Protocol for Microcentrifuge (QIAGEN). The purified PCR product was sequenced using the same primers as those in the PCR reactions and BigDye Terminator Kit (v3.1) by using the cycle sequencing technology (dideoxy chain termination / cycle sequencing) on ABI 3730XL sequencing machines (MWG). Multiple alignments were done by using the online version of multiple alignment program for amino acid or nucleotide sequences (MAFFT version 6).

3. RESULTS

3.1. Haplotypes

All samples showed the typical PCR-products ranging between 571 bp and 838 bp corresponding to the predicted $P_o$and Q repeat pattern in the target region. Restriction with the enzyme *Dra* I yielded seven different restriction patterns (Fig. 2) matching the haplotypes A_1, A_4, A_8, A_{13}, O_1, O_1' and C_2 (Garnery et al., 1993, 1995; Franck et al., 1998, 2000a, 2000b, 2001). Figure 2 shows acrylamide gels with the types and fragment patterns of the samples in wild populations (A) and the apiaries (B). Five haplotypes: A_1, A_4, O_1, O_1' and C_2 were confirmed by sequence analyses.

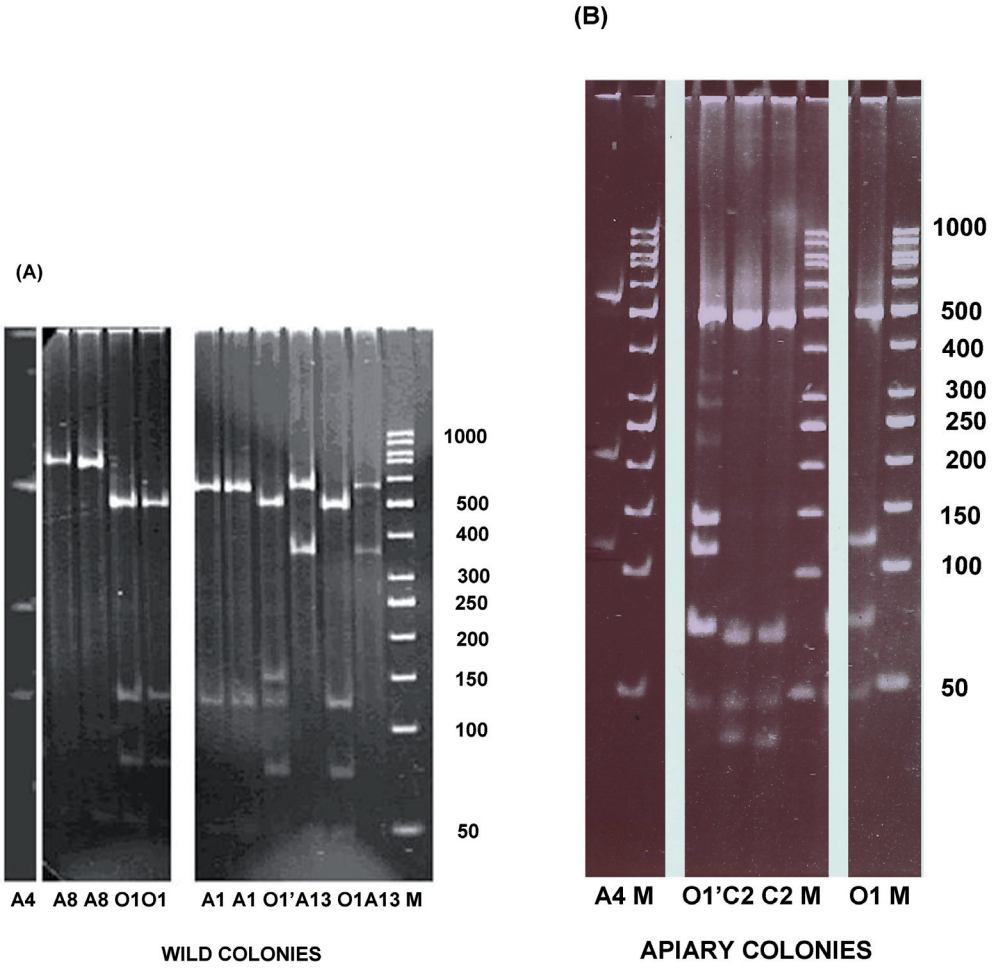

Figure 2. MtDNA fragment patterns of honey bees of Sudan in a 10% acrylamide gel after digestion with the enzyme *Dra*I. Six different restriction patterns were found corresponding to the haplotypes: A_1 (47 bp; 108 bp; 483 bp), A_4 (47 bp; 108 bp; 193 bp; 483 bp), A_8(47 bp; 591 bp), A_{13} (47 bp; 310 bp; 483 bp), O_1(47 bp; 67 bp; 108 bp; 420 bp), O_1' (47 bp; 67 bp; 67 bp; 108 bp; 129 bp; 420 bp), C_2 (47 bp; 64 bp; 420 bp). The haplotypes A_1, A_4, O_1, O_1' and C_2 were confirmed with sequencing. M = weight marker (100 bp ladder). The occasional mismatch artefacts of the observed restriction fragment pattern with the expected ones were confirmed by sequencing the PCR products.

3.2. Spatial distribution of haplotypes

The distribution of haplotypes per sample location is given in Table I. whereas the distribution of lineages per sample location is given in Figure 1. The honeybees sampled in wild colonies either belonged to the A or the O lineage. The O_1 haplotype was present in all the surveyed localities except South west (11) and South Sudan (12, 13). Particularly high O_1 frequencies were observed in central Sudan and along the northern part of the Nile valley (1, 2, 3, 5, 9), whereas the O_1' haplotype was frequent in east Sudan (3, 4, 6, 7). The A_4 and A_1 haplotypes were frequent in west (10, 11) and south Sudan (12, 13) States. The haplotypes A_8 and A_{13} are restricted to the south-west (10) and south-east (7) respectively. Only

the colonies sampled on commercial apiaries (2, 6) included the non-native haplotype C_2 in addition to the native types.

There was a clear co-segregation of lineages and ecosystems. Honeybees of the wet savannahs and forest ecosystems all showed the A lineage, which is typical for *A. m. scutellata* and *A. m. adansonii*. In contrast, all samples from wild colonies in desert, semi-desert and dry savannah areas had the O lineage similar to *A. m. lamarckii* and *A. m. syriaca.* Mixed populations with A and O lineages were found in the transitional zones between wet and dry ecosystems and on the commercial apiary in Khartoum (2).

4. DISCUSSION

Clearly, we did not find any Sudanese honeybees that belonged to the Y lineage. This does not exclude that they might exist in non-sampled regions, but it does suggest that this lineage may be rare at best in Sudan. The high frequency of the O lineage in Sudan shows that a third lineage of *Apis mellifera* mtDNA is endemic to north-eastern Africa. Given this high mtDNA diversity and three lineage branches radiating from this region, our results support Ruttner et al.'s (1978) theory that north-eastern Africa and the Near East (Mashriq) might be a centre of origin of *A. mellifera*, which then spread north and south. They proposed that the species invaded Africa and Europe in three distinct branches, a South and Central African branch (A), a North African and West European branch (M) and a North Mediterranean branch (C). This classification was further refined by adding the fourth biogeographic branch, O, including the Near- and Middle-Eastern subspecies (Ruttner, 1988) and later confirmed by mtDNA data (Smith and Brown, 1988; Garnery et al., 1992, 1993; Arias and Sheppard, 1996; Franck et al., 2000b; Franck et al., 2001).

We only found honeybees carrying A lineage haplotypes in the southern and south western parts of Sudan. According to Franck et al. (2001), the lineage A is composed of three sublineages; the first group, A_I, (A_1–A_4, A_6, A_{12}, A_{13}, A_{19}, and A_{24}–A_{27}) is endemic to most of Africa south of the Sahara. A second group, A_{II}, is characterized by haplotypes A_8, A_{10}, corresponding to *A. m. sahariensis* and *A. m. intermissa* from the Maghreb countries in Northern Africa. The third sublineage is characterized by haplotypes with the P_1 sequence (group A_{III}) typical to *A. m. iberiensis* populations in Portugal and the Canary Islands. In our samples we found group A_I haplotypes including A_1, A_4, A_{13} and the A_8 haplotype belonging to the A_{II} group. The high frequency of group A_I haplotypes in the west and south are in line with Franck et al. (2001), who report on a progressive decline of haplotype A_1 frequencies from Guinea towards south-eastern Africa to be eventually replaced by haplotype A_4 in *A. m. monticola*, *A.m. scutellata* and *A. m. capensis.*

Haplotypes A_8 and A_{13} are typical of *A. m. intermissa* in Northern Africa (Garnery et al., 1995; Franck et al., 2001). We found these types in two wild colonies, in an area lacking any beekeeping activities. Since apiculture had no impact on these populations, the bees must have come there by natural means. Although these were only single colonies, our sampling was coarse and population frequencies may actually be considerably high. We cannot exclude that the occurrence of these haplotypes in Sudan may reflect ancient migration processes during the Middle Holocene (~8000 years BP) from northern Africa into Sudan or the opposite (Gasse et al., 1990; Hooghiemstra et al., 1992; Ritchie, 1994) when the Sahara was a savannah-type habitat. The long range seasonal migrations typical of African honeybees of the savannahs may have facilitated the spread of honeybees across the now desert regions. A similar population admixture is also observed in northern Africa, where group A_I haplotypes are found together with group A_{II} populations (Franck et al., 2001).

Honeybees in the highlands of Ethiopia and Kenya are morphometrically (Amssalu et al., 2004) and genetically (Meixner et al., 2000) different from honeybees in the lowland and savannah. If the Y lineage is typical to the mountain highland populations of Ethiopia, the lack of this type in Sudan may be due to the low altitudes. Sudan is mostly flat land with a mean altitude of 500 m, and

hence one would not expect any ecotypes typical of a highland habitat. Because Sudan lacks major geographical barriers separating honeybee populations, the differences in climate and vegetation seem to be the major cause of honeybee subspecies diversification (Potts and Behrensmeyer, 1992). Climatically there are four major zones in Sudan (van Chi-Bonnardel, 1973; Walter, 1976; Rudloff, 1981): hot desert, subdesert or Sahel, dry tropical and wet tropical. Correspondingly, the vegetation of Sudan has been classified into different types from north to south following the climatic zones (Harrison and Jackson, 1958; El-Sarrag et al., 1992; El-Sarrag, 1977; Hepburn and Radloff, 1998): desert, semi-desert, dry-savannah, wet-savannah and forests (Tab. I, Fig. 2). The desert regions cover about one third of the entire country with only very light and irregular rain fall (0–50 mm per year). There is no vegetation, except in desert valleys and adjacent to the River Nile. Further south, the semi-desert region is richer in vegetation due to 50–300 mm rainfall per year, followed by the dry savannah region with an annual rainfall of 300–500 mm per year and a dry season of four to six months (starting in April). The wet savannah region includes Bahr Elarab, Jebel Marra, Nuba Mountains, Ingessana Hills and White Nile tributaries with annual rainfalls of up to 1000 mm. Finally, forests form the most southern vegetation belt in the Sudan with annual rainfalls of up to 1600 mm resulting in thick vegetation.

The biogeographical distribution of A and O lineages of honeybees fits well with the climatic differentiation of Sudan. The O lineage was primarily observed in the dry regions, whereas the A lineage is more typical of the tropical climate (Garnery et al., 1993, 1995; Franck et al., 1998, 2000a, b, 2001). Even within the lineage A haplotypes of South and South-West Sudan, the ecosystem classification seems to hold. Type A_1 honeybees primarily occurred in the wet savannahs and forests regions, the typical biome for *A. m. adansonii*. In contrast, the haplotype A_4 was only observed in dry savannahs, the typical biome of *A. m. scutellata* (Ruttner, 1988). In general, the transitional zones between African honeybee subspecies are associated with transitions between ecosystems (Hepburn and Radloff, 1997) which nicely fits with the transition from the A- to the O lineage in Sudan.

According to our analyses, the honeybees in Sudan appear to be composed of two lineages, A and O, with different haplotypes O_1, O_1', A_1, A_4, A_8, A_{13} rather than one lineage (Y). Clearly, honeybees of the dry regions are genetically more similar to *A. m. lamarckii*, whereas the honeybees of the wet regions are more similar to *A. m. adansonii*. Hence the honeybees of Sudan may not represent a single well defined subspecies but may rather reflect a mix of O and A lineage populations with a strong difference between bees in the North and in the South. This may explain the problems experienced in morphometrical studies where morphoclusters were often incoherent and did not result in concise classifications (Hepburn and Radloff, 1996, 1997).

In addition, we found local evidence of introgression of commercially imported honeybee stock. Although this is of principle concern from a conservation point of view, it seems to have had no far reaching impact yet, because it was confined only to the few commercial apiaries sampled. Given the lack of European haplotypes in the wild colonies, in spite of repeated and massive introductions over many decades (Mogga, 1988; El-Sarrag and Nagi, 1989), this may be another case where natural selection favours locally adapted *A. mellifera* and purges maladapted imported stock from the population (Moritz et al., 2005).

ACKNOWLEDGEMENTS

Research was funded by the EC within the FP 6 Integrated Project "ALARM" (Assessing LArge scale environmental Risks for biodiversity with tested Methods; GOCE-CT-2003-506675 (Settele et al., 2005) and the Strategic Research Project BEE SHOP (Bees in Europe and Sustainable Honey Production) (RFAM). Financial support was also granted by the Deutsche Akademische Austauschdienst and the National Centre for Research, Khartoum, Sudan (MAAE).

Caractérisation des abeilles (*Apis mellifera*) du Soudan par l'ADN mitochondrial.

***Apis mellifera jemenitica* / ADN mitochondrial / race / discrimination / biogéographie / Soudan**

Zusammenfassung – Mitochondrielle Unterscheidung der Honigbienen (*Apis mellifera*) Sudans. Die Klassifizierung der Honigbienen (*Apis mellifera*) Sudans wird kontrovers diskutiert. Sie wurden zunächst als *A. m. nubica*, dann als *A. m. jemenitica* und schließlich als Gruppe heterogener Morphotypen klassifiziert. Die Honigbienen Sudans sind von einer Reihe verschiedenster Bienenrassen umgeben, die unterschiedlichen biogeographischen Linien angehören: Im Norden die O-Linie, im Osten die Y-Linie und im Süden und Westen die A-Linie. Angesichts dieser großen Variation, sollten mtDNA Polymorphismen ein ideales Werkzeug sein, um den Subspezies-Status der sudanesischen Honigbienen zu überprüfen. Die Größe des Landes und die extreme Ökosystemvielfalt stellt günstige Vorbedingungen für eine große Honigbienenvielfalt. Wir nutzen mtDNA-Polymorphismen, um die Subspezies-Zugehörigkeit zu bestimmen und zu prüfen, inwieweit durch Bienenimporte und Imkerei nichtendemische Rassen in wilde Populationen eingedrungen sind.

Arbeiterinnen wurden von 47 wilden und 28 imkerlich bewirtschafteten Völkern an verschiedenen Standorten (von Wüste bis Regenwald) im Sudan gesammelt (Abb. 1). DNA wurde extrahiert und die mtDNA mit PCR Methoden amplifiziert. Die PCR Pordukte wurden mit *Dra* I restringiert und die Restriktionsmuster im Acrylamidgel visualisiert (Abb. 2). Alle Haplotypen wurden zur Bestätigung des jeweiligen mtDNA-Typus sequenziert.

Wir fanden keine Y-Haplotypen, die als typisch für *A. m. jemenitica* gelten. Statt dessen fanden wir sieben verschiedene Haplotypen aus der A- und O-linie (Abb. 1) und *A. m. jemenitica* scheint im Sudan keine häufige subspezies zu sein. Die hohe Diversität scheint in erster Linie durch die sehr unterschiedlichen Ökosystemtypen des Landes bestimmt zu sein, da die Haplotypen eng an die klimatischen Bedingungen gekoppelt sind. Die Bienen der feuchteren Klimate zeigten den A_1 und den A_4 Typus (Abb.1) während Bienen aus den trockeneren Gegenden der O-Linie (O_1 und O_1') zugehören. Der C_2-Haplotyp kam nur in einer Imkerei mit rezent importierten Bienenköniginnen europäischer Herkunft vor. In den Wildpopulationen konnten keine Hinweise auf nichtendemische Honigbienen gefunden werden.

Die Reklassifikation der sudanesischen Honigbienen hat weitreichende Konsequenzen für die Interpretation der Biogeographie von *A. mellifera* im Maghreb und Mashriq. Die Häufigkeit des O-Typus im Sudan bestätigt erneut, dass im Nordosten Afrikas drei verschiedene biogeographische Linien zusammentreffen. Die These von Ruttner et al. (1978), der Nordosten Afrikas stelle eine Region mit hoher Variabilität dar, findet somit erneut Unterstützung.

***Apis mellifera jemenitica* / Bienenrasse / mitochondrielle DNA / Sudan / Biogeographie**

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