Genetic Variability of the Mitochondrial DNA in Honeybees (*Apis mellifera* L.) from Benin

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**Abstract:** The aim of this study was to evaluate the genetic variability in bees *Apis mellifera* from Benin by using mitochondrial DNA (mtDNA) as a molecular marker in their cytochrome c oxidase subunit I and II (*COI-COII*) intergenic region. A total of 304 bee colonies were sampled in 27 municipalities of the cashew growing area of Benin. These samples were analyzed by the cleaved amplified polymorphisms technique for determining the haplotypes of subspecies present in the sampled population. Eight PCR-RFLP profiles of African lineage A were then identified in the 304 samples of bees investigated. Forty-nine percent (49%) of the samples showed the profile of haplotype A1 (subspecies *adansonii* of Zambia), 40% of haplotype A4 (subspecies *scutellata* of South Africa) and 3% of haplotype A19 (subspecies *adansonii* of Guinea). Five other haplotypes of the African branch (A) that had been described in a previous study were also identified: new 1 (2%), new 2 (2%), new 3 (1%), new 4 (2%) and new 5 (1%). This study showed that *A. mellifera* from Benin belonged only to lineage A with the predominance of haplotypes A1 and A4. This study will contribute to the development of coherent policies for conservation of local bees in Benin.

**Key words:** *Apis mellifera adansonii*, mitochondrial DNA, cytochrome c oxidase subunit I and II, African lineage, Benin.

1. Introduction

*Apis mellifera*, an endemic bee in Africa, Europe and Asia, is one of the arthropods species of great agricultural and ecological importance. In fact, in consuming pollen and nectar, bees pollinate many species of wild and grown plants. Apart from the production of honey, royal jelly and wax, bees contribute to over 80% of pollination services in global agriculture [1, 2], estimated at 153 billion euros per year [3]. In recent decades, a multifactorial gradual decline has been observed with bees, facilitated by climatic and human disturbances, biological invasions, import of non-local subspecies, parasites and pathogens.

These arthropods can be classified according to morphological and molecular criteria. Morphometry has long been the only way to describe the genetic diversity of the honeybee in Africa. According to Latreille [4] and David [5], the subspecies *adansonii* was the one described for both West and Central Africa. Equally, Hounkpè et al. [6] described the same subspecies in Northern and Central Benin. However, based on the morphometric characteristics, three distinct groups of bees were identified in the North-East and South of Benin [7-9]. Recent studies have shown that bees from Benin are divided into 10 statistically distinct morphotypes [10, 11].

The molecular characterization of bees then seems
necessary, because the morphometric approach has
enough limits [12]. Morphometry has also been a very
powerful way, since it has not only enabled the
distinction of subspecies and branches. Only developed
molecular markers, including mitochondrial DNA
(mtDNA) and microsatellites can help validate and
extend the knowledge of the biodiversity of the species.

One of the most reliable methods for the molecular
characterization of bees is sequencing their
mitochondrial genome [13]. So, using these tools, five
lineages (A, M, C, Y and O) of honeybees have been
described based on the genotypic characters of the
whole mtDNA [14-17] and the high variability of the
cytochrome c oxidase subunit I and II (COI-COII)
region [18]. The size of the region COI-COII depends
on its structure. Differentiation in variations in the size
of the DNA fragments helps to identify the
“evolutionary mitochondrial” lineages and to reveal
the polymorphisms between lineages [14, 19, 20]. The
COI-COII region is made up of the combination of
two types of sequence P/Po and Q (Q, P/PoQ, P/PoQQ
and P/PoQQQ) [14]. This interesting tool is not used
enough in the characterization of bee colonies in West
Africa and particularly in Benin. So, it appears
necessary to identify the different subspecies of the
African lineage (A) present in Benin with a view to
develop coherent policies for preserving local bees.
Hence, the present work aimed at analyzing the
mtDNA of bee colonies of Benin.

2. Materials and Methods

2.1 Sampling

Samples of bees were collected in the three
agro-ecological zones (Center, North-East and
North-West) that are propitious for the growing of
cashew in Benin [21]. In these three zones, 27
municipalities in all were selected in the study based on
the abundance of both cashew plantations and honey
farms (Fig. 1). A total of 304 bee colonies was
collected as follows: 105 colonies in the North-West,
100 in the Centre and 99 in the North-East. Before
capturing the bees, the holes of the selected colonies
were previously smoked out in order to reduce their
aggressiveness. Ten bees per colony were captured in
boxes containing 96% ethanol. Once in the laboratory,
the sampled bees were individually put into Eppendorf
tubes, containing 1.5 mL of 96% ethanol and stored at
4 °C before molecular analysis.

2.2 Molecular Characterization of Bees

2.2.1 DNA Extraction

One bee per colony was dried in the open air and
the head was dissected. Then, the total DNA was
extracted from the head using Chelex extraction
method [22]. After the extraction, the DNA was stored
in a freezer at -20 °C until use.

2.2.2 PCR Amplification of the COI-COII Region
and Gel Electrophoresis

The extracted DNA was used for amplifying the
intergenic region COI-COII of the mtDNA, using
primers E2 (5’-GGCAGAATAAGTGCATTG-3) and
H2 (5’-CAATATCATTGATGACC-3), according to
the protocol of Garnery et al. [14]. DNA amplified was
electrophoresed, using 1.4% agarose gel
 electrophoresis based on protocol of Alburaki et al.
[17].

2.2.3 Restriction Polymorphism Analysis

To know the variability of the COI-COII region in
Benin bees, the PCR amplicons were cleaved by the
Dr al (AAA/TTT) restriction using the protocol of
Garnery et al. [14]. The product of restriction was
loaded on a 7.5% polyacrylamide gel based on protocol
of Alburaki et al. [17]. The different profiles of
PCR-RFLP obtained were used to characterize the
polymorphism in the bee’s population [14, 19, 20].
Unbiased estimates of haplotype diversity [23] and the
corrected haplotype diversity were calculated based on
the total number of haplotypes in the population.

2.2.4 Statistical Analysis

The results of each colony were recorded in the
Excel 2010 spreadsheet for determining the
membership lineage of the colonies analyzed. The total
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Fig. 1 Geographical location of bee-collecting areas of Benin.
of all haplotypes per zone was calculated. Then, the proportion of each haplotype with regard to the total of all haplotypes per zone was also calculated. The three-sample test for equality of proportions without continuity correction followed by proportion structuring tests was carried out to evaluate the existence or not of significant difference among zones and to identify areas with similar proportion in case of significant difference among zones.

In each population, two types of software (PowerMarker version 3.2 and Gene GenAlEx version 6.5) were used to estimate haplotype frequencies. The index of genetic diversity was determined using the formula of Nei [23] with GeneAlEx version 6.5. The genetic distance computed based on the different haplotype profiles obtained was used for a binary matrix to construct a dendrogram through the N algorithm.

3. Results

3.1 Fragment Length Variability in the COI-COII Intergenic Region

All samples showed typical PCR products ranging between 571 bp and 1,375 bp, corresponding to the predicted Po and Q repeat patterns in the target region in bees (Fig. 2). The vast majority was the PoQ length (51.31% PoQ, 43.42% PoQQ and 5.26% PoQQQ).

3.2 Identified Haplotypes

Restriction with the Dral enzyme resulted in eight different restriction patterns, matching haplotypes A1, A4, A19 and five new ones named new 1, new 2, new 3, new 4 and new 5, which have not yet been described in previous studies of bees in the world.

Fig. 3 showed the picture of acrylamide gels with the haplotypes obtained. The five new haplotypes obtained are haplotypes A1 and A4 with the deletions in their bases sequences.

3.3 Frequency of the Different Haplotypes across the Study Area

Fig. 4 showed the distribution of the different haplotypes across the study area. There is a predominance of haplotype A1 (48.68%) followed by haplotype A4 (40.46%). The five new haplotypes represented less than 10%.

3.4 Distribution of Haplotypes across Zones

The results of the three-sample test for equality of proportions without continuity correction revealed significant difference ($p < 0.001$) in the proportions between haplotypes A1 and A19 (Table 1). Also, a significant difference was observed in the proportions between A4, new 2 and new 4 among the study areas ($p < 0.05$). No significant difference was observed between haplotypes A12, new 2 and A9. Among the studied zones, tests revealed structuring proportions indicating that, even if there were significant differences among the zones for haplotypes A1, the proportion of the Central zone and that of the North-West zone are the same. The same result was
| Haplotypes | A1 | Sin 2-1, Sin 2-4, Bem 3-4, Cen 1-1 | None | NIK 3, TCH 13, GLA 17 |
|------------|----|-----------------------------------|------|----------------------|
| A4         | PAR 2-1, PAR 1-1, Sin 3-1, Sin 1-1, Ber 2-1, Ber 1-1, Bem 3-1, Par 2-4, Par 1-4, Sin 3-4, Sin 1-4, Ber 2-4, BER 1-4, FAK 1-1, Nag 2-1 | BEM 1-1, FAK 2-4, BAS 2-4, CEN 2-4, NAG 3-4, MAN 3-4, BOU 1-4, BEM 1-4, FAK 3-1, BAS 3-1, NAG 1-1, MAN 1-1, COP 1-1, BOU 2-1, ATA 3-1, BEM 2-1, FAK 3-4, BAS 3-4 | DER 1, DAL 11, PER 11, SAL 3, SID 2, KET 2 |
| A19        | None | None | None | NAT 10, NAT 8, NAT 5 |
| New 1      | None | m ATA 2-2, m ATA 2-9, ATA 2-1, ATA 2-4 | None | None |
| New 2      | None | None | None | DAL 17, PER 20, DER 2, SID 6 |
| New 3      | None | None | None | SID 10, SID 14 |
| New 4      | None | None | None | PER5, NIK 7, BIR 1 |
| New 5      | BAS 1-1 | None | None | PER 3 |
| Ladder     | Marq V, M4, A10 | Marq V, M4 | Marq V | |

Fig. 3  Picture of acrylamide gels showing the haplotypes obtained.
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Fig. 4  Distribution of the different haplotypes across the study area.

Table 1  Distribution of haplotypes across zones.

| Zone       | A1   | New 1 | A19  | A4   | New 3 | New 2 | New 4 | New 5 |
|------------|------|-------|------|------|-------|-------|-------|-------|
| Centre     | 66.67%<sup>a</sup> | 3.57%<sup>a</sup> | 0%<sup>b</sup> | 28.57%<sup>b</sup> | 0%<sup>a</sup> | 0%<sup>b</sup> | 1.19%<sup>b</sup> | 0%<sup>a</sup> |
| North-East | 32.17%<sup>a</sup> | 1.74%<sup>a</sup> | 0%<sup>b</sup> | 51.30%<sup>a</sup> | 1.74%<sup>a</sup> | 4.35%<sup>a</sup> | 6.09%<sup>a</sup> | 2.61%<sup>a</sup> |
| North-West | 52.38%<sup>a</sup> | 0%<sup>a</sup> | 7.62%<sup>a</sup> | 38.10%<sup>b</sup> | 0.95%<sup>a</sup> | 0%<sup>b</sup> | 0%<sup>b</sup> | 0.95%<sup>a</sup> |

Probability: 0.000 0.158 0.000 0.005 0.471 0.015 0.012 0.258

Significance: *** ns *** ** ns ** ns

Proportions with the same letter in the same column are not significantly different. *** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \), ns: \( p > 0.05 \).

3.5 Phylo-geographical Relationship between Haplotypes through the Neighbour-Joining Method

Fig. 5 showed the phylo-geographical relationship between the haplotypes through the neighbour-joining method. In general, the analysis of the phylogenetic tree showed a mixture of the samples collected throughout the study area. Thus, it appears that the distribution of the population genotypes is not based on their collection zones. For example, Djidja’s (Central zone) ecotypes and Manigri’s (North-West zone) ecotypes have the same genetic distance and are in the same group. However, it was observed that the North-East and North-West ecotypes within the populations of Boukoumbé, Béroubouay, Copargo and Parakou are grouped together.

3.6 Distribution of Haplotypes across Zones

The principal component analysis, which was performed by considering the samples of regions as individual groups P1, P2 or P3 in Fig. 6 showed an information value of 67% on the first two axes. On these two axes, all genotypes were grouped into three groups G1, G2 and G3 (Fig. 6), characterized by:

First group (G1): composed of genotypes from the three different regions with a predominance of samples from the Central and North-West regions;

Second group (G2): composed of three genotypes of populations, mostly collected in the North-West and North-East regions;

Third group (G3): consisted only of genotypes from two zones (North-West and North-East). In these two zones, no haplotype from the Central zone was identified.

Overall, the principal component analysis (PCA) did not specify any haplotypes according to sampling regions (Fig. 6).
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Fig. 5  Phylo-geographical relationship between haplotypes through the neighbour-joining method.

Fig. 6  PCA showing the distribution of haplotypes across different regions.
4. Discussion

This study, which aimed at analyzing the mtDNA of bees from Benin helped to identify the different subspecies of bees of the lineage A present in Benin. The restriction profiles that were obtained were analyzed and compared with the existing mtDNA markers. The results showed the presence of eight haplotypes of lineage A (A4, A1, A19) and five other ones named new 1, new 2, new 3, new 4 and new 5, never being described in previous studies. These results corroborate those of Garnery et al. [14, 24], which indicate that lineage A is most suited to the tropical climate. However, a study conducted by Franck et al. [25] shows that the evolutionary A lineage consists of three sub-lineages characterized by the presence and frequency of particular haplotypes and their geographical distribution. Thus, the first A1 group, consisting of haplotypes A1, A4, A6, A12, A13, A19, A27 and A24 is endemic to most parts of sub-Saharan Africa. The second AII group made up of haplotypes A8, A9 and A10 corresponds to *A. m. sahariensis* and *A. m. intermissa* from North African countries. The third sub-lineage consists of haplotypes with the P1 sequence (group AIII) that is specific to populations of *A. m. iberiensis* in Portugal and the Canary Islands. Haplotypes A1, A4 and A19 obtained in the study belong to group A1 according to the classification described by Franck et al. [16].

The high frequency of haplotypes A1 and A4 in the population of the three study areas corroborates the results of Franck et al. [16], which indicate that African bee colonies mainly present haplotypes A1 and A4.

The distribution of haplotypes in the areas showed that the two new haplotypes new 2 and new 3 identified are especially found in populations of the North-West and North-East. This could be justified by the fact that these two areas are in regions bordering Burkina Faso and Niger and could also explain the high variability of haplotypes observed in the two areas. However, no significant difference was observed between haplotypes in the three areas.

The presence of haplotypes A1 in all populations from the Central area could be explained by the fact that haplotype A1 is the mitotype, which is characteristic of the *adansonii* subspecies and was originally described for all of West and Central Africa by Latreille [4]. Several studies conducted on the morphometry of bees in Benin also confirmed the presence of this subspecies in Central and Northern Benin [6, 9-11].

Haplotypes A1, A4 and A19 correspond to specific subspecies of the African lineage, namely *A. mellifera* (Zambia), *A. m. scutellata* (South Africa) and *A. m. adansonii* (Guinea) [16]. The presence of eight haplotypes in the study environment suggests a cohabitation of a lot of subspecies in Benin. The predominance of haplotype A1 in the North-West (66.67%) and Centre (52.40%), and the A4 haplotype in the North-East (55.60%) indicates that the subspecies *A. m. adansonii* is characteristic of North-West and Central Benin while *A. m. scutellata* is characteristic of the North-East.

The results of the phylogenetic tree analysis showed a mixture of samples collected throughout the study area. The grouping of North-East and North-West ecotypes among populations of Boukoumbé, Béroubouay, Copargo and Parakou on the phylogenetic tree could be justified by a gene flow due to the migration of bees from North-East to North-West and vice versa. The PCA specified no haplotype according to the sampling areas. These data corroborate the results of Paraïso et al. [9] on the study of morphometric characterization of bees from Benin. Indeed, those authors indicate the presence of three morphotypes that are mostly a mixture of honeybees from several municipalities. This also corroborates the results of the study by Hepburn and Radloff [26], who reported a frequent maternal fusion of honeybee colonies in tropical Africa. The variability in the haplotypes observed in the three agro-ecological zones
confirmed the results of several authors, who showed
the presence of three very distinct morphological
groups within the bee populations of Benin [7, 9]. The
lack of bees, belonging to the evolutionary lineages M,
C, O and Y in the sampling suggests that there have
been no importations of queens from these lineages by
Benin beekeepers, which is important for conservation
of local bees in Benin.

5. Conclusions

Bees from Benin belong to the African lineage;
results confirmed that the original haplotypes in
Africa are those of mtDNA lineage A. The
polymorphism which is observed among haplotypes is
due to the coexistence of several subspecies that are A.
m. adansonii, A. m. scutellata and other subspecies in
Benin. The existence of five new haplotypes (new 1,
new 2, new 3, new 4 and new 5) paves the way for
further research. Taking into account the results of this
study will allow better conservation of bees and
knowledge of the different haplotypes present in Benin.

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