

Africanization of Melliferous Bees (*Apis mellifera*.L.) Bibliographic Review

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Abstract

Context: The hybridization process (Africanization) of European bees with African bees is a problem for apiculture farmers in the Americas, due to high swarming levels and defensiveness. The latter hinders colony handling, and has caused accidents to people and animals, increasing the risks of beekeeping. In this sense, there is a need for genetic breeding of melliferous bees, and previous identification of subspecies.

Aim: To evaluate the origin of melliferous bees (*Apis mellifera*), and the process of Africanization and dispersion of Africanized bees throughout the Americas, as well as methods of identification.

Methods: The databases of Scencedirect, Google-Scholar, Scopus, and NCBI were reviewed under the following key words, *Apis mellifera*, *Apis*, Africanized bees, geometric morphometrics, mitochondrial DNA. Special emphasis was paid to papers published within the last five years.

Results: The origin and distribution of melliferous bees, and the Africanization and dispersion processes of Africanized bees were described. Additionally, the evolution of methods for the characterization of *Apis mellifera* species were updated.

Conclusions: Africanization can be considered the most important process in the transformation of conduct and morphological features of melliferous bees, which allowed for their rapid dispersion in the Americas. The identification methods based on parents are essential to know possible process of genetic erosion, and to present strategies for bee conservation and breeding in every region.

Key words: *mellifera*, *Apis*, Africanized bees, geometric morphometrics, mitochondrial DNA.

Introduction

Africanization is one of the fastest and most striking biological invasions ever known worldwide (Branchiccela et al., 2014; Medina-Flores et al., 2015), and there are divergent criteria in favor and against the results for apiculture in the Americas. Several actions are being taken to control this process, such as the introduction of European germplasm, and colony isolation, though neither has stopped these effects. Accordingly, learning to live with these bees seems to be the logical solution, since these species have positive traits, such as a marked hygienic behavior, high production of honey, and so on. Then, it is important to characterize bees to learn the existing genetic material in every region, and set actions in the mid and long terms, in order to reduce

defensiveness and swarming, which are negative traits found in the Africanized hybrids. The rationale for these arguments aims to examine the origin of melliferous bees (*Apis mellifera*), the process of dispersion of Africanized bees through the Americas, and the methods of bee identification.

Development

Origin and distribution of *Apis mellifera*

The evolution of class *Insecta*, and order *Hymenoptera* took place in the Jurassic period, approximately 180 millions of years ago, as a result of the co-evolution of flower plants, which had ovaries and stigmas, and depended on insects for entomophile reproduction-pollinizing (Carpana,

2004). The diversity of species is the result from radiation in the early Pleistocene period due to climatic change and habitats during the last ice age (Combey, Quandahor & Mensah, 2018).

Of the three subfamilies of the Apidae family (Meliponinae, Bombinae, and Apinae), Apinae withstood the best adaptation, which made them cosmopolitan (Verde, 2014). *A. mellifera* is the only bee species that evolved in Europe, the Middle East, and Africa, where several subspecies can be identified (Genchi, Reynaldi & Bravi, 2018). Depending on the geographical origin, there are 29 subspecies (Valido, Rodríguez-Rodríguez & Jordano, 2014; Abizanda, 2018) with remarkable morphological and genetic differences (Wallberg et al., 2014), with the largest distribution worldwide, and tropical and temperate ecotypes.

Subspecies are grouped in five evolutionary lines, mainly determined by the climatic and flora variations, as a result of glacier and post-glacier periods (Miguel et al., 2016).

Meixner et al. (2013) noted that line A includes African subspecies *A. m. intermissa* and *A. m. sahariensis* in the North; *A. m. lamareckii*, *A. m. simensis*, and *A. m. adansonii* in the west and center; *A. m. scutellata* in the center and south; *A. m. capensis* in the south, *A. m. monticola* in the mountainous region of southeastern Africa, *A. m. litorea* in the southeast of Africa, and finally *A. m. unicolor* which is endemic to Madagascar (Techer et al., 2017). Line M is made of Western Europe subspecies, including *A. m. mellifera*; *A. m. iberiensis* (Kandemir, Özkan & Fuchs, 2011). Line C is made of Eastern Europe subspecies, including *A. m. ligustica*, *A. m. carnica*. Line O includes the Middle East subspecies (Rúa, Martínez, Domingo & Gabaldón, 2013; Charistos, Hatjina, Bouga, Mladenovic & Maistros, 2014; Wallberg et al., 2014). And Y includes subspecies *A. m. jemenitica* from Ethiopia (Meixner et al., 2013). Line Z, with populations

spread in Libya (Péntek-Zakar, Oleksa, Borowik & Kusza, 2015)

In the sixteenth century, the European settlers introduced melliferous bees (*A. mellifera*) in the Americas; the data show the dispersion process in North America in 1622, Cuba, 1763, and Brazil and Chile 1839 (Nogueira-Neto, 1972). In Ecuador, the first bee-hives were introduced in 1870 with the honey producing European ecotype *A. m. ligustica* (Hidalgo & Mena, 2003).

In the second half of the twentieth century, in most of the Americas, a biological invasion known as

Africanization took place (Kerr, 1967). This phenomenon (Garcia et al., 2013) resulted from the escape of African bees (*Apis mellifera scutellata*) from an experimental beekeeping facility in Brazil, in 1957.

The Africanization of melliferous bees in 1956, *A. m. scutellata*, African line, was introduced in Brazil from South Africa and Tanzania (Chapman et al., 2015), which started as a breeding project to increase honey production of European bees in the tropical climate of the Amazon basin. The European bees are unable to link their biological cycle to the duration of the day in the tropic. However, flowering in temperate areas is framed, and in the tropics, flowering responds to rainy periods (Moore, Wilson & Skinner, 2015).

The idea was to create a “super bee”, a hybrid between European and African subspecies with proper temperament and search habits (Whitfield et al., 2006).

Nevertheless, Africanized bees have a high genetic variability due to their hybrid character; they are also known by the high productivity and tolerance against pathogens and parasites like *Varroa destructor* (Strauss, Dietemann, Human, Crewe & Pirk, 2015; Tibatá et al., 2018).

Factors like genetic plasticity and adaptability of bees (Sousa, Araújo, Gramacho & Nunes, 2016), and the wide geographical dispersion and environmental variability in the Americas, allow for hybrid evolutionary processes (Nunes, Araújo, Marchini & Moreti, 2012). By being in contact, the African and European colonies, the process of hybridization lead to the replacement and displacement of European traits by African ones (Rangel et al., 2016). Africanized bees are characterized by their high defensive behavior (DB), and migration (Medina-Flores et al., 2015). Their propagation causes problems to apiculture, and had important social and economic impacts due to their high defensiveness (Byatt, Chapman, Latty & Oldroyd, 2016).

Today, Africanized bees are present from the mid and south of the United States (Szalanski & Tripodi, 2014; Portman, Tepedino, Tripodi, Szalanski & Durham, 2018), Central America to the north of Argentina (Genchi et al., 2018), covering 20 million km² approximately. Their high colonizing activity is one of the fastest biological invasions known (Branchiccela et al., 2014; Medina-Flores et al., 2015).

Jarnevich et al. (2014) noted that hybrid Africanized bees spread out, and continue to do so in the continent (**Figure 1**). The first swarms of African

bees arrived in Ecuador, Colombia, and Peru in the 1970s (Graciano, 2018).

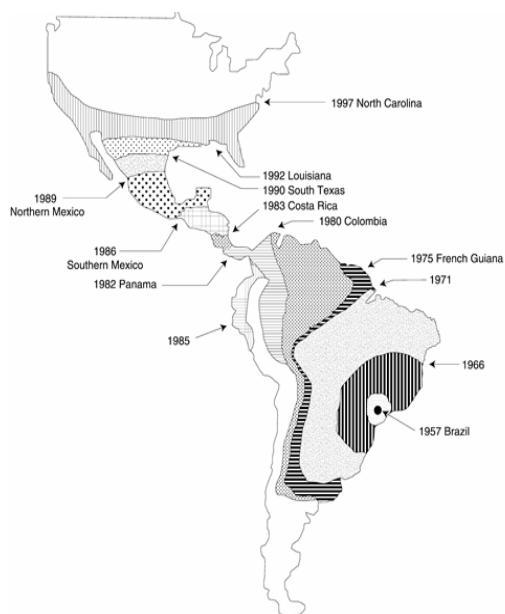


Fig. 1. Dispersion of Africanized bees in the Americas (Winston, 1992).

Wallberg et al. (2014) claimed that Africanized bees in South America are mostly from African parents (70-90%), and in the United States (50-75%). This distribution probably shows the adaptation to local environmental conditions, and hybridization between melliferous bee subspecies (Coroian et al., 2014). The Africanization level may have implications in adaptability, biological attitude, behavior, pollinizing, and production of honey by *A. mellifera* (Mendoza et al., 2014; Hamiduzzaman et al., 2015). In this sense, the conservation of the genetic diversity of melliferous bees is important to preserve the high genetic adaptability of populations in different zones (Büchler et al., 2014), which is closely related to them.

Evolution of characterization methods of subspecies *Apis mellifera*

Since many years ago, several methods are being used to identify and classify *A. mellifera*, one of them is traditional morphometrics (Alpatov, 1929; Ruttner, 1988). Moreover, quite a few methods for morphological and molecular analysis are used for identification of *A. mellifera* hybrid, such as the Fast Africanized Bee Identification System (FABIS). This method permits preliminary identification of Africanized bees (Sylvester & Rinderer, 1987).

The Universal System to Detect Africanization (USDA-ID) used to declare official cases of Africanization (Sanford, 2006), is painstaking, since it requires twenty-five body parts or structures from each specimen. The Automated Bee Identification

System (ABIS) that compares wing-vein graphs with a digital image from the anterior part of the sample, requires two minutes per sample, with an estimated confidence of 98.05% between species, and 94% between subspecies of melliferous bees (Francoy et al., 2008).

Currently, geometric morphometrics methods have shown significant results in classification and identification of insects. This method provides a powerful tool to study the biological and phylogenetic classifications (Su, Cai & Huang, 2018). The geomorphometric methods can be used to solve taxonomy problems, and to visualize key differences in the shape of species (Canal, Hernández-Ortiz, Salas & Selivon, 2015; Qubaiová, Růžička & Šípková, 2015).

The classification of different lines is supported by more accurate methods, such as biochemical methods: isoenzymes and aloenzymes (Nunamaker & Wilson, 1982; Ivanova et al., 2012); nuclear DNA molecular markers (Charistos et al., 2014); mitochondrial DNA (Szalanski & Tripodi, 2014); sequencing (Branchiccela et al., 2014; Kono & Kohn, 2015); microsatellites (Rangel et al., 2016), and cuticle hydrocarbons (Oyerinde, Salako & Rabiou, 2017). In recent years, the utilization of single nucleotide polymorphisms (SNP) has become

relevant (Chávez-Galarza et al., 2015; Chapmaneta, 2015; Muñoz et al., 2015).

Morphometrics

The concept of beekeeping taxonomy was introduced by Dupraw (1965), and it was developed by Ruttner, Tassencourt & Louveaux (1978).

The first subspecific classifications were based on geographical and conduct features, depending on their distribution (Ruttner et al., 1978; Ruttner, 1988). Using morphometrics, diagnostic methods can be utilized in multiple measurements of many individuals; in physical and structural variations, which are universal features of a population, genus, species or subspecies (Darger, 2013). Now, subspecies differentiation is based on morphometric characteristics (Kandemir et al., 2011; Kono & Kohn, 2015; Sousa et al., 2016), which undergo gradual changes between stages, especially between subspecies that often just have a slight difference in the mean values (Abizanda, 2018).

Traditional morphometrics

The traditional morphometrics allowed for identification and classification of *A. mellifera*

(Daly, Hoelmer, Norman & Allen, 1982; Ruttner, 1988). Variance and covariance studies of distance are used between point pairs, usually length and width of structures, in addition to proportions and angles (Rohlf & Marcus, 1993). The morphometric characteristics of bees are used, on the other hand, to identify breeds or geographical subspecies. Among the first morphometric classification techniques are the ones established by Cochlov, in 1916, cited by Ruttner (1988), who compared proboscis length among six geographically different breeds of *A. mellifera*.

Later, new sets of characteristics were introduced by Goetze, in 1940, like hair length in the terga, the width of the first tarsus segment, and the length diameter of terga 3 and 4 (Ruttner, 1988), which were very efficient for *A. mellifera* subspecies discrimination. Howell, Daly & Balling (1978), developed the first efficient procedure, which consisted in measuring 25 characters by hand: wings, sternite, and legs, using an optical micrometer, with reliable results. These measurements were analyzed through multivariate statistics for identification. Later, Daly et al. (1982), improved this method with the inclusion of digital measurements (measurement system linked to a computer), that led to a much easier and faster procedure.

Ruttner (1988) perfected this technique, and described 36 morphometric features (distances, angles, pigmentation classes) for the discrimination of melliferous bees subspecies. These features helped establish four different evolutionary lines of melliferous bees (Ruttner, 1992).

The traditional morphometrics includes wing and body features that offer substantial understanding and proper discrimination among bee subspecies and populations. In the search for method improvements, 10 features described by Ruttner, in 1988 were used (Meixner et al., 2013).

The efficiency of this classification depends on the selection of relevant features that prevent mistaken information (da Silva et al., 2015). However, it is still used in various research to characterize subspecies in locations such as the mountain forest of Crimea (Bykova, Triseleva, Ivashov & Safonkin, 2016), in the Republic of Benin (Amakpe et al., 2018), Colombia (Graciano, 2018).

Geometric morphometrics

With the transition from descriptive to quantitative morphometrics, morphological identification is the most accurate and reproducible, since it relies on computerized techniques (Adams, Rohlf & Slice, 2004). The descriptive analysis of size and shape variations is a fundamental tool for organic biology studies (De Souza et al., 2015), which became

popular in the late twentieth century, with the fusion of biology and geometry, in order to collect geometric information from biological forms, for comparative purposes (Torcida & Perez, 2012).

From the beginning, geometric morphology (GM) showed better descriptive power, in addition to an approach that offers a relative description of the form by using reference points (Santana et al., 2014). It relies on integrated statistical analysis to collect spatial information of morphological structures, thus increasing accuracy in relation to traditional morphometry (Francoy, de Faria Franco & Roubik, 2012). It allows for specific analysis of morphometric variations of particular structure in organisms with different sizes (Nunes et al., 2012).

Geometric morphometry uses the coordinates of reference points, which are then overlapped through displacement, scale-up, and rotation, to remove the size effect (da Silva, Grassi, Sella, Francoy & Reali, 2015). Following overlapping, the landmark configurations only differ in their shape, and can be analyzed through multivariate statistical methods (Zelditch, Swiderski, Sheets & Fink, 2004).

Variation in the shape of the wing of bees at different taxonomic levels, with the utilization of GM is useful to discriminate subspecies (Charistos et al., 2014; De Souza et al., 2015; Oleksa & Tofilski, 2015; Abizanda, 2018), and the geographical variation of species (Hall, MacLeod & Wardhana, 2014; Carvajal et al., 2015). Furthermore, since the morphometric characteristics of bees are highly inherited, this technique can be suitable for contrasting.

GM was highly effective to discriminate 24 known subspecies of melliferous bees in Europe (Kandemir et al., 2011). In turn, Oleksa & Tofilski (2015) indicated that GM provides similar discrimination values to microsatellites. The high accuracy of classification indicates that the front wings carry sufficient information to distinguish the groups of bees examined (Charistos et al., 2014). Besides identifying hybridization process, the morphometric techniques are related to nuclear microsatellite analysis that measure changes in the size and shape of bee bodies, since they are controlled by nuclear genes (parent inheritance), in keeping with the arguments of Mortensen & Ellis (2015).

They also allow for optimization of analysis time by reducing the number of individuals used; 10-15 bees are recommended for morphometric analysis (Kandemir et al., 2011; Meixner et al., 2013; Sousa et al., 2016). The number of individuals may be reduced to five, to analyze more stable traits, such as wing veins (Francoy et al., 2008), with a high level of consistency between the wing and molecular information.

Molecular techniques

The different molecular analyses performed to study the genetic diversity of *A. mellifera*, provide an accurate tool compared to classic morphometrics (Szalanski & Tripodi, 2014). Melliferous bee hybrids are morphologically similar, and hard to distinguish from one another, so in some cases molecular analysis is necessary (Achou et al., 2015). In the last 35 years, the identification and distribution of different *A. mellifera* lines based on molecular and mitochondrial markers has been reported, (Wallberg et al., 2014) (Table 1)

Table 1. Recent molecular biology research to identify the maternal origin of melliferous bees

Analysis	Genes	Subspecies	Author
Analysis of polymorphism restriction	tRNAle u-COII, Cyt b, rRNA – COI, COI	<i>Apis mellifera</i>	Wu, Lu & Lu (2017)
Analysis of polymorphisms and sequencing	tRNAle u- COII	<i>Apis mellifera</i>	Branchiccola et al. (2014)
Sequencing	mtDNA	<i>A. m. scutellata</i> <i>A. m. capensis</i>	Eimanifar, Kimball, Braun & Ellis (2018)
Sequencing	COI - COII	<i>Apis mellifera</i>	Magnus & Szalanski (2010)
Sequencing	COI - COII; ND2	<i>A. m. unicolor</i>	Techer et al. (2017)
Analysis of polymorphism restriction	COI - COII	<i>Apis mellifera</i>	Kelomey et al. (2017)
Analysis of polymorphism restriction	COI - COII	<i>A. m. intermissa</i> <i>A. m. sahariensis</i>	Achou et al. (2015)
PCR - RFLP	Cyt b	<i>A. m. scutellata</i>	Mortensen & Ellis (2015)
Sequencing	tRNAle u- COII	<i>Apis mellifera</i>	Szalanski & Magnus (2010)
Sequencing	COI - COII	<i>Apis mellifera</i>	Techer et al. (2015)

Sequencing	tRNAle u- COII	<i>A. m. iberiensis</i>	Chávez-Galarza et al. (2017)
Sequencing	mtDNA - Cyt-b	<i>Apis mellifera</i>	Szalanski & Tripodi (2014)

Mitochondrial DNA

The first molecular studies relied on the study of highly variable mitochondrial intergene COI-COII

locus (Péntek-Zakar et al., 2015); they were started in

the early 1990s (Garnery, Solignac, Celebrano & Cornuet, 1993). It is a frequently used tool to discriminate evolutionary lines in melliferous bees populations (Rortais, Arnold, Alburaki, Legout & Garnery, 2011).

Mitochondrial DNA (mtDNA) is a tiny, 16 000 base pairs, circular molecule, which is completely transmitted by the queens to their offspring: workers and drones (Meixner et al., 2013). The mother inheritance of mtDNA in the bees is a molecular marker that allows for tracing the origin and genetic structure of melliferous bees. The study of a single individual provides direct information from the hive (Rúa et al., 2013), since this circular molecule is not changed by recombination, and it is transmitted without altering the offspring, except in cases of punctual mutations.

The mitochondrial genome is characterized by conserved gene contents, high level of nucleotide substitutions, and as a provider of a single parent

marker (Péntek-Zakar et al., 2015). The

mitochondrial chromosome of *A. mellifera* contains 13 genes that codify for proteins, two ribosomal genes, 22 transfer RNA, and a region which is rich in A + T. It is conservative in terms of their position (Crozier & Crozier, 1993).

The intergene COI-COII locus is made of two distinct nucleotide sequences, named P and Q, where P can appear in several variations (P0, P1, P2). The variations observed in the sequences of the region can be used to contrast the five lines of melliferous bees, and to discriminate the subspecies of *A. mellifera* from one another (Alattal et al., 2014). According to Garnery et al. (1993), each evolutionary line includes a P sequence variant combined with one of the four copies of the Q sequence found in that locus, except for the C line subspecies, which do not have a copy

of the P sequence, and only possess a Q sequence copy (Rúa et al., 2013).

The codifying insert of the intergene locus helps distinguish the African line from the European one, since the melliferous bees of the east European line (C) have a shorter intergene sequence due to the absence of P, and a single copy of Q (Meixner et al., 2013).

Melliferous bees that belong to lines M, A, Z, and Y have longer intergene regions, because they contain between one and five Q elements (Rortais et al., 2011), in addition of a variant of the P element.

The mitochondrial chromosome of *A. mellifera* (Figure 2) contains 13 genes that codify for proteins, two ribosomal genes, 22 transfer RNA, and a locus that is rich in A + T. It is conservative in terms of their position. Both features are stable in evolutionary terms (Crozier & Crozier, 1993).



Fig. 2. Mitochondrial DNA scheme of *Apis mellifera* (Crozier & Crozier, 1993)

There is a variety of molecular methods used to detect mtDNA variations, perhaps RFLP (restriction fragment length polymorphisms), PCR-RFLP, and direct sequencing. Several genetic classifications of *A. mellifera* were based on RFLP and PCR assays, where the non-coding locus between tARNLeu and COII was amplified by PCR, followed by with *DraI* endonuclease digestion, with identifiable band patterns due to differences in size (Garnery et al., 1993).

The utilization of PCR-RFLP allows for the identification of more than 100 haplotypes properly allotted to their evolutionary lines (Rúa, Jaffé, Dall'Olio, Muñoz & Serrano, 2009). However, despite the greater resolution of the *DraI* test in relation to other tests, it is unable to make distinctions between the C and O lines, which results in a mistaken classification, since it fails to identify melliferous bees at the level of subspecies, because it

does not allow for identification of haplotypes in the diagnostic (Meixner et al., 2013).

Genotyping by sequencing amplified fragments in the COI and COII intergene loci shows a greater level of genetic variation among different lines; therefore, they constitute a molecular marker that provides higher accuracy in the detection of mitotypes that represent specific genetic variations (Tibatá et al., 2018). They generate identical haplotypes by offspring, instead of identical haplotypes by state, which is necessary for phylogenetic analysis.

Sequencing and characterization of mtDNA genome is useful to analyze the phylogeny and genetic structure of the population of *Apis* genus. Today, mtDNA assays of the COI – COII intergene locus are done to characterize *A. mellifera* subspecies in the Americas (Branchiccela et al., 2014; Szalanski & Tripodi, 2014; Tibatá et al., 2018), en Africa (Eimanifar, Kimball, Braun & Ellis, 2016; Haddad et al., 2017), Europe (Ostroverkhova et al., 2015; Chávez-Galarza et al., 2017), and Asia (Zhao et al., 2014; Takahashi et al., 2016; Wu et al., 2017). The COI-COII intergene locus is widely used as a tool to detect and trace progressive import of melliferous bee queens (Muñoz, Lodesani, Rúa & Dall'Olio, 2014), in phylogeographical analysis in native areas, and identification and understanding of secondary contact

areas (Chávez-Galarza et al., 2015).

Nowadays, the novel techniques used for DNA sequencing include single nucleotide polymorphic markers (SNP) (Henriques et al., 2018). This technique has become more popular in recent years (Pinto et al., 2014), and it is very effective to separate bee lines (Harpur et al., 2014). Muñoz et al. (2016) when comparing microsatellite and SNP tests based on high performance sequencing, demonstrated that SNP is more accurate for the identification of European subspecies. Moreover, the greatest advantage is that the frequency and dispersion of Africanization can be known.

However, a few years ago, low cost molecular assays to detect Africanization were unavailable. Whitfield et al. (2006) clearly distinguished Africanized bees from European bees using 1136 SNPs; Chapman et al. (2015), using a 95 SNP ancestral informative panel could identify Africanization in populations of commercial bees in the United States and Australia with a high confidence level, through offspring assignment. Today, low cost SNP tests are suggested.

However, since mtDNA is inherited from the mother, mtDNA markers cannot determine if a European queen was mated by African drones, and if these

colonies will remain undetected by mtDNA techniques (Szalanski & Tripodi, 2014). Accordingly, mtDNA assays are recommended for studies to identify subspecies to trace the maternal origin of colonies; father contribution can be detected using nuclear introgression indicators (Kono & Kohn, 2015).

Conclusions

Africanization can be considered the most important process in the transformation of conduct and morphological features of melliferous bees, which allowed for their rapid dispersion in the Americas. The identification methods based on parents are essential to know possible process of genetic erosion, and to present strategies for bee conservation and breeding in every region.

Author contribution

Diego Masaquiza: research planning, data collection, analysis and interpretation of results, redaction of the manuscript, and final review.

Lino Miguel Curbelo Rodríguez: research planning, data collection, analysis and interpretation of results, redaction and final review of the manuscript.

Amilcar Arenal: research planning, data collection, analysis and interpretation of results, redaction and final review of the manuscript.

Conflicts of interest

No conflict of interest has been declared.

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