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Original article

Hybrid status of honey bee populations near the historic origin of Africanization in Brazil

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Summary — Africanized honey bee populations are genetically heterogeneous across their extensive new world range. Over 35 years have elapsed since the introduction of *A m scutellata* to southeastern Brazil and we hypothesized that populations from this region should have achieved the highest degree of genetic equilibrium following the perturbation of introduction. We report here the results of a population genetic study of honey bees sampled near the origin of neotropical Africanization combining analyses of morphological, allozyme and mtDNA characters. Data from this study support previously reported allozyme frequency estimates and support the expectation that populations from this region are comparatively stable in genetic composition; and further, that significant polymorphism of European origin persists in the Africanized population of the region. Morphological and mtDNA data from these neotropical populations reveal the strong influence of the African race, *A m scutellata.* Apparent discordance among data sets from the several analytical methods reflects variation in selection and population size on the inheritance or persistence of such characters and indicates the importance of multiple character analysis.

Africanized honey bee / Brazil / population genetics / morphometry / mt DNA / enzyme polymorphism

INTRODUCTION

Over two dozen subspecies of the honey bee, *Apis mellifera,* occur throughout its old world range (Ruttner, 1988). A subset of these races has been introduced to the new world over the past 4 centuries (Kerr, 1967; Morse *et al,* 1973; Sheppard, 1988), although the effective sampling of races for some of the introductions was quite limited (Aeppler, 1922; Sheppard, 1988). Despite the varied African, European and Middle Eastern origins of introduced subspecies, it is generally held that new world feral and managed honey bee populations were largely or wholly of European derivation prior to the mid 1950s (Michener, 1975; Taylor, 1985; Rinderer, 1986). At that time the South and Central African honey bee race, *Apis mellifera scutellata* Lepeletier was introduced to tropical Brazil through both the inadver-

tent release of *scutellata* queens and deliberate distribution of African x European racial hybrids to improve Brazilian beekeeping (Kerr, personal communication, in Spivak *et al,* 1991). Prior to the release of *A m scutellata,* neotropical *A mellifera* populations were poorly adapted to tropical ecological and climatological conditions and existed primarily in managed apiaries. The density of European-derived feral colonies during that period was apparently quite low (Michener, 1975; Taylor, 1988). However, the success of the African honey bee introduction can be measured in the extensive and rapid range expansion that followed. Within 20 years the descendent "Africanized" honey bees reached their southern limits in Argentina (Kerr *et al,* 1982), while their northward expansion continues in the southern US more than 35 years after the initial Brazilian introduction.

A number of hypotheses have been proposed regarding the mechanisms underlying "Africanization". These range from suggestions that genetic contributions by both races produce more or less "hybrid" populations, with the relative advantage of either race being dependent upon a number of ecological or behavioral factors (Kerr and Bueno, 1970; Michener, 1975; Rinderer *et al,* 1985; Rinderer, 1986) to the notion that expanding neotropical populations are maintained as a relatively pure African gene pool through natural selection, the effects of pre-mating isolating mechanisms or hybrid inviability (Taylor, 1985; Fletcher, 1991). Consequently, the genetic composition of Africanized honey bee populations, vis-à-vis the relative contribution of progenitor European and African races has been a matter of considerable research and discussion. Despite differences, one generalization possible from the body of research is that Africanized honey bee populations appear heterogeneous throughout their geographical range. For example, Africanized honey bee populations near their climatic limits in the temperate zone (Sheppard *et al,* 1991) or in recently colonized neotropical regions (Del Lama *et al,* 1990) containing a substantial pre-existing European population (Rinderer *et al,* 1991) can exhibit significant amounts of both African and European allozyme or mtDNA characters and hybrid morphologies. Established neotropical Africanized populations show a lesser influence of European races based on similar characters or nuclear DNA markers (Lobo *et al,* 1989; Hall, 1990; Moritz and Meusel, 1991) or virtually none at all (Hall and Muraldiharan, 1989; Smith *et al,* 1989). When considered in the context of the current geographical range of bees with African-derived genetic characteristics, the heterogeneous nature of new world Africanized honey bee populations is not an unexpected result, although the phenomenon of Africanization and the "Africanized" population has sometimes been more narrowly defined (Hall, 1990). As the geographic range of the Africanized honey bee has expanded, the most recently occupied regions undergo a transition to Africanization over a period of at least several years (Boreham and Roubik, 1987; Taylor, 1988; Rinderer *et al,* 1991). Given that the number of generations following contact between African- and European-derived populations differs throughout the extant range of Africanization, one might therefore predict the region of initial contact in southeastern Brazil to have achieved the greatest equilibrium. Recent analysis of honey bees from this region, in contrast to an earlier report (Smith *et al,* 1989), found evidence of prior hybridization between the 2 groups, based on the presence of a presumptive European mtDNA marker, although the population was morphometrically indistinguishable from African *A m scutellata* (Moritz and Meusel, 1991). A large-scale allozyme

study also revealed that populations of this region express significant levels of European-derived introgression, although morphometrically they are quite "African" (Lobo *et al*, 1989). In this paper we report the relative genetic contribution of African- and European-derived subspecies to Brazilian honey bee populations near the original site of introduction of *A m scutellata*, based on a combined analysis of mtDNA, morphological and allozyme character data.

MATERIALS AND METHODS

Samples of honey bees were collected from 126 colonies at 4 locations within the state of Sao Paulo, Brazil. These locations were all within 200 km of the initial release site of *A m scutellata* in Rio Claro, Brazil (Kerr, 1967) and at least 60 km from each other (fig 1). The locations and number of colonies sampled were as follows: Ribeirao Preto, SP (13), Luis Antonio, SP (14), Santa Rosa do Viterbo, SP (33), and Ibitiuva, SP (66). All colonies originated as collected swarms maintained without subsequent queen management practices, and therefore are considered representative of local feral populations. Samples of adult workers were collected alive and then frozen in liquid nitrogen for later mtDNA and allozyme analyses or stored in EtOH for morphometric study.

Allozyme analysis

Ten individuals per colony were characterized for 3 enzyme loci, Mdh-1, Pgm-1 and Hex-1, known to be polymorphic in honey bees. Gene

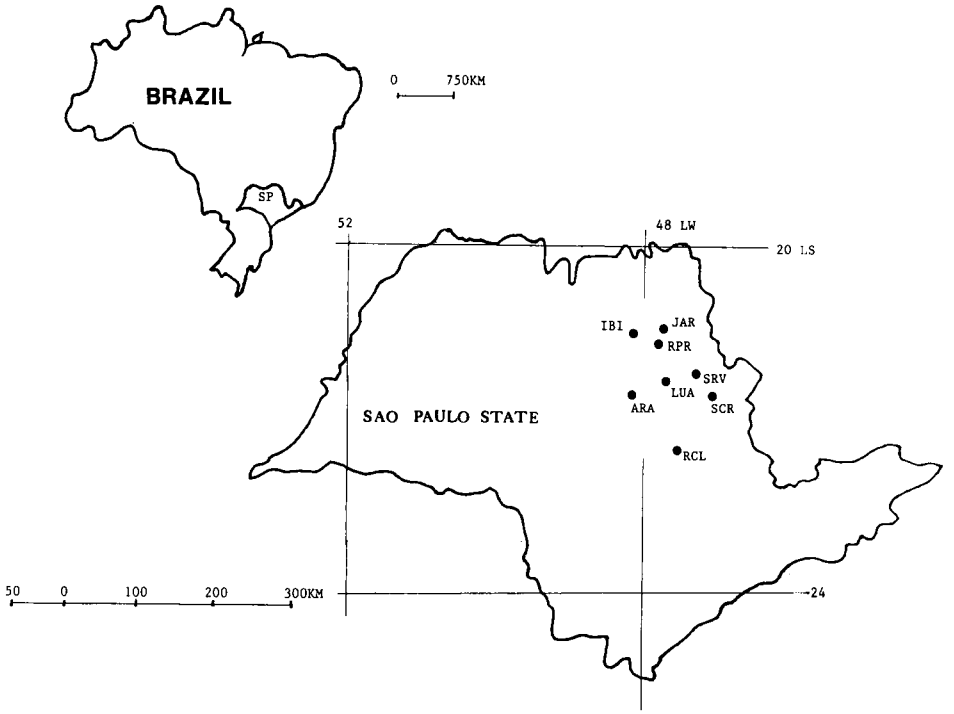

Fig 1. Honey bee collecting sites within São Paulo State, Brazil. ARA: Araraquara; IBI: Ibitiuva; JAR: Jardinopolis; LUA: Luis Antonio; RCL: Rio Claro; RPR: Ribeirao Preto; SCP: Santa Cruz das Palmeiras; SRV: Santa Rosa do Viterbo.

frequencies at these loci have been widely used in studies of Africanization and racial hybridization in the honey bee (Cornuet, 1982; Sylvester, 1982; Badino *et al*, 1983; Sheppard and McPheron, 1986; Spivak *et al*, 1988; Lobo *et al*, 1989; Smith *et al*, 1989; Del Lama *et al*, 1990). Homogenates of individual bees were run on standard starch gel conditions and visualized using appropriate histochemical techniques (Mdh-1 and Pgm-1; Sheppard and Berlocher, 1984; Sheppard and McPheron, 1986; Hex-1: Del Lama *et al*, 1988). Allozymes were scored using relative mobilities and gels were photographed for documentation. Analysis of allozyme data was performed with the Biosys-1 program of Swofford and Selander (1981).

Mitochondrial analysis

Total nucleic acids were extracted from 2 individuals/colony, generally following the method of Sheppard and McPheron (1991). Restriction digests were made on aliquots of the resuspended total nucleic acids according to conditions specified by the supplier (Bethesda Research Laboratories, Bethesda, MD). All samples were digested with *Eco*-R1 and *Bcl*-1, enzymes that have been used to provide diagnostic identification of African mtDNA (Smith, 1988; Hall and Muralidharan, 1989; Sheppard *et al*, 1991; Rinderer *et al*, 1991). Samples were run on 1% agarose gels overnight at 24 V and nucleic acids transferred to nitrocellulose filters using standard techniques (Southern, 1975).

Purified mitochondrial DNA was prepared from fresh honey bee flight muscle as follows: 8 g of degastered adults were homogenized on ice in a modified Erway grinder in 2-g aliquots with 40 ml of mitochondrial isolation medium (MIM) buffer (220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 1 mM EDTA, 1 mM EGTA; pH 7.5). Mitochondria were isolated through a series of differential centrifugations and then lysed with 1% SDS for 5 min at 25 °C. Protein was precipitated with cesium chloride (vol x 0.22 g/ml) on ice for 15 min and pelleted at 12 000 *g*. The density of the supernatant was adjusted with additional cesium chloride to 1.63 g/ml and the mixture was placed into a Beckman quick-seal tube. Ethidium bromide was added (1 mg/tube) and the sealed tubes were centrifuged in a Beckman 80 Ti rotor at 50 k rpm for 18–24 h. The resulting band of purified closed circular mtDNA was removed and repurified with an additional CsCl density gradient centrifugation (Beckman SW65 rotor) at 40 k rpm for 18–24 h. The repurified mtDNA band was removed, treated with butanol to remove ethidium bromide and then drop-dialyzed (Marusyk and Sergeant, 1980) to remove the salt. Radioactive probe was produced from this purified honey bee mtDNA using standard nick translation conditions (Maniatis *et al*, 1982).

Prehybridization and hybridization of nitrocellulose filters occurred at 50 °C with standard solutions containing salmon sperm DNA and 25% formamide (Maniatis *et al*, 1982). Hybridized filters were washed, dried and placed on X-ray film for 6–24 h for autoradiography. Fragment sizes were estimated from the autoradiographs using a digitizer and Bioscan software.

Table I. Allozyme frequencies of 4 honey bee populations sampled in 1990 near origin of Africanization in Sao Paulo State, Brazil.

Site *	*No col*	*Allozyme frequency*								
		Mdh^{100}	Mdh^{80}	Mdh^{65}	Hex^{100}	Hex^{83}	Pgm^{100}	Pgm^{82}	Pgm^{75}	Pgm^{50}
RPR	13	0.79	0.14	0.07	0.43	0.57	0.92	–	0.06	0.02
LUA	14	0.84	0.12	0.04	0.40	0.60	0.95	–	0.04	0.01
SRV	33	0.81	0.15	0.04	0.46	0.54	0.91	0.01	0.08	–
IBI	66	0.82	0.14	0.04	0.43	0.57	0.83	–	0.13	0.04

* RPR : Ribeirao Preto; LUA : Luis Antonio; SRV : Santa Rosa do Viterbo; IBI : Ibitiuva.

Table II. MDH allozyme frequencies published for populations of Africanized honey bee from Sao Paulo State, Brazil *.

Site **	No col	Allozyme frequency		
		Mdh^{100}	Mdh^{80}	Mdh^{65}
LUA	25	0.79	0.20	0.02
SCP	35	0.78	0.17	0.05
JAR	21	0.77	0.17	0.06
RCL	64	0.78	0.18	0.04
ARA	40	0.78	0.19	0.03

* Lobo *et al,* 1989; Del Lama *et al,* 1990. ** LUA : Luis Antonia; SCP : Santa Cruz das Palmeiras; JAR : Jardinopolis; RCL : Rio Claro; ARA : Araraquara.

Morphometric analysis

Ten bees from each sample were dissected, stained and slide mounted as described by Daly and Balling (1978). For each bee, 25 characters were measured using a microscope slide projector and Houston Instruments digitizer. The resulting data were analyzed with a discriminant analysis program in widespread use for Africanized honey bee identification (Daly and Balling, 1978). Samples were scored as "Africanized" or "European" based on at least 98% probability of group membership. *A m scutellata* is not readily discriminated from neotropical Africanized honey bees by this program, although it can detect Africanized populations containing genetic contributions from any of several European races (Sheppard *et al,* 1991). Samples that were outside of the 98% probability criteria were scored as "non-discriminated".

RESULTS AND DISCUSSION

Results of the allozyme survey are summarized in table I. The mean frequency of the Mdh^{100} allele, commonly believed to be fixed (freq = 1.0) or nearly so in *A m scutellata* (Nunamaker and Wilson, 1981; Sylvester, 1982; Smith *et al,* 1989; Lobo *et al,* 1989) was 0.82, similar to previous estimates from large-scale studies of Sao Paulo honey bee populations (table II) and identical to that reported from Rio Claro, the initial site of African honey bee release (Lobo *et al,* 1989). The mean frequency for the Hk^{83} allele, reported only from African-derived populations (Del Lama *et al,* 1988; Spivak *et al,* 1988) and variation at the Pgm locus were remarkably similar to previous studies of populations from this region (Lobo *et al,* 1989; Del Lama *et al,* 1990) and indicates the degree of genetic equilibrium of this population some 35 yr after Africanization. Contingency table analysis of heterogeneity among populations, performed with the HETXSQ option of BIOSYS, revealed non-significant heterogeneity for all loci among the populations (P = 0.83, 18 df). Inclusion of published data from Sao Paulo State (table II) gave similar results for the MDH locus (P = 0.99, 16 df) and supports the existence of extensive homogeneity among these 9 Brazilian populations.

Mitochondrial analysis of the colonies produced the restriction fragment patterns "typical" of *A m scutellata* (Smith, 1988). An additional *Eco*-R1 polymorphism reported from Argentine and Mexican Africanized honey bees (Rinderer *et al,* 1991; Sheppard *et al,* 1991) and known to occur in the honey bees of Spain (Sheppard, unpublished observations) was not found. Recently Smith *et al* (1991) have reported that some colonies of honey bees collected in southern Spain, considered to be the race *A m iberica* based on geography, share a mitochondrial *Eco*-R1 polymorphism with *A m scutellata* and many Africanized populations. However, they were able to differentiate these Spanish honey bees from *A m scutellata* by screening with an additional enzyme, *Hinf*-1. Although we

did not examine our Brazilian samples with *Hinf*-1, analysis of over 30% of them with a 1.4 kb honey bee mtDNA probe cloned from the A+T-rich region revealed a *scutellata*, rather than *A m intermissa* or *iberica*, origin for the mtDNA in our samples (SSP-1 digestions of honey bee total nucleic acid extractions probed with this fragment produce fragment patterns diagnostic for several African subspecies, including *A m scutellata;* Sheppard, unpublished observations).

Morphometric analysis of the colonies determined all samples to be Africanized with a probability of at least 0.98. The overall discriminant score for the collection was 4.18 (SD = 1.04). In 125 out of 126 cases, colony discriminant scores were over 2.16, the score that defines the 0.99 level of probability for determining Africanized honey bees (S Buco, personal communication).

As a group the 4 populations of honey bees were quite "Africanized" based on morphology and all individuals exhibited "African" mtDNA. However, allozyme data from these populations are in accord with previous studies indicating that Brazilian Africanized honey bees are descended from both African and European progenitors (Lobo *et al,* 1989; Del Lama *et al,* 1990; Moritz and Meusel, 1991). Such seemingly incongruous conclusions in population genetics can perhaps be best explained by differences in inheritance and selective parameters influencing the various genetic markers (Sene *et al,* 1988). Thus, while allozymes are presumed to be largely neutral, with respect to selection (Berlocher, 1984), morphology may be more closely linked to physiological and behavioral traits of tropical and temperate adapted African and European races, respectively. In the neotropics then, traits advantageous to tropical survival and expressed by *A m scutellata* might be at a selective advantage.

Extranuclear markers with specific patterns of inheritance, while also assumed to be selectively neutral, may be eliminated from populations through their association with specific selected genotypes. Thus, in the case of maternally inherited European mtDNA, the genetic composition of colonies produced by European virgin queens mated with Africanized drones approaches 50% African-derived genes as a maximum. In the neotropics, these colonies may be at such a competitive disadvantage compared to colonies headed by African queens (which approach a maximum of 100% African-derived genes) that selection could be a very strong barrier to the entry of European mtDNA into the feral population. However, such hybrid colonies would still provide an early source for entry of European genes into the feral population, through mating by their drones and, thus, may be important in contributing European-derived allozyme genes to Africanized populations such as found in this study. In locations with reduced selection for tropical genotypes, such as the temperate regions of Argentina, hybridization leads to the dissemination of mtDNA into the "opposite" morphologies on either side of the hybrid zone (Sheppard *et al,* 1991; Sheppard, unpublished observations).

The validity of using African and European mtDNA frequencies to assess the genetic composition of Africanized honey bee populations rests on prior knowledge, or at least reasonable estimates, of relative population sizes (Page, 1989). While the population of European colonies in neotropical Brazil was apparently small, relative to the quickly-established feral AHB population, over 1 million European-derived colonies, part of an extensive beekeeping industry, was present in the Yucatan of Mexico prior

to the arrival of AHB to that region. Rinderer *et al* (1991) reported that neotropical Africanized populations collected from this region several years after Africanization express high levels of European mtDNA. Thus, both natural selection and relative population size may play roles in the ultimate genetic composition of Africanized honey bee populations.

Based on the data presented herein on feral honey bees sampled near the geographic origin of Africanization in the new world, and genetic investigations from similar sites in Sao Paulo State, Brazil (Lobo *et al,* 1989; Del Lama *et al,* 1990; Moritz and Meusel, 1991), it is evident that Africanized honey bees from these long-established populations express substantial mtDNA and morphological similarities to the tropical race, *A m scutellata,* while still retaining evidence of prior hybridization. The compositional flux or stability of these populations as they become established in temperate North America will certainly be a matter of great interest to population geneticists and apiculturists alike.

Résumé — Statut hybride des populations d'abeilles près du lieu d'origine de l'africanisation au Brésil. Les populations d'abeilles africanisées sont génétiquement hétérogènes dans toute leur aire de répartition dans le Nouveau Monde. Plus de 35 ans se sont écoulés depuis l'introduction d'*A m scutellata* dans le sud-est du Brésil et nous émettons l'hypothèse que les populations de cette région ont atteint le plus haut degré d'équilibre génétique après la perturbation due à l'introduction. Nous donnons ici le résultat d'une étude de génétique des populations d'abeilles échantillonnées près de la source de l'africanisation néotropicale, réalisée en combinant les analyses des caractères morphologiques avec ceux des allozymes et de l'ADN mitochondrial (ADNmt).

Les échantillons d'abeilles ont été prélevés dans 126 colonies en 4 endroits de l'état de São Paulo, Brésil. On a caractérisé 10 individus par colonie à l'aide de 3 locus d'enzyme, *Mdh*-1, *Pgm*-1 et *Hex*, connus pour leur polymorphisme chez l'abeille. Les analyses morphométriques de 10 individus par colonie ont été faites en suivant la procédure de Daly et Balling (1978). L'ADNmt de toutes les colonies a été caractérisé par les digestions par les enzymes de restriction *Eco*R1 et *Bcl*-1 des extractions totales d'acides nucléiques et par électrophorèse sur gel d'agarose. Les fragments de restriction ont été visualisés par hybridation avec une sonde «nick-translatée» faite à partir d'ADNmt purifié d'abeilles, suivie d'autoradiographie.

En tant que groupe, les 4 populations d'abeilles sont, d'après leur morphologie, très «africanisées» et toutes possèdent un ADMmt «africain». Pourtant, les données des allozymes provenant de ces populations concordent avec les précédentes études qui indiquaient que les abeilles brésiliennes africanisées descendent à la fois de parents africains et européens (Del Lama *et al,* 1990; Lobo *et al,* 1989; Moritz et Meusel, 1991). L'homogénéité des estimations de la fréquence des allozymes parmi les populations étudiées ici et précédemment indiquent que les populations de cette région ont une composition génétique relativement stable. Les données de la morphologie et de l'ADNmt de ces populations néotropicales montrent une forte influence de la race africaine, *A m scutellata.* La discordance apparente entre les ensembles de données issues des diverses méthodes analytiques reflète la variabilité des influences de la sélection et de la taille de la population sur la transmission ou la persistance de tels caractères et montre l'importance d'une analyse par des méthodes variées.

abeille africanisée / Brésil / génétique des populations / morphométrie / ADNmt / polymorphisme enzymatique

Zusammenfassung — Hybridzustand von Honigbienenpopulationen nahe dem historischen Ursprung der Afrikanisierung in Brasilien. Die Populationen von afrikanisierten Honigbienen sind heterogen über ihrem gesamten weiten Verbreitungsgebiet in der Neuen Welt. Seit der Einführung von *A m scutellata* nach Südostbrasilien sind jetzt über 35 Jahre verstrichen und wir stellen die Hypothese auf, daß die Populationen aus dieser Region nach den Störungen der Einführungszeit jetzt den höchsten Grad eines genetischen Gleichgewichtes erreicht haben sollten. Wir berichten hier über die Resultate einer populationsgenetischen Studie an Bienenproben, die nahe dem Ursprung neotropischer Afrikanisierung gesammelt wurden. Dabei wurden Analysen von morphologischen, Allozym- und mtDNA-Merkmalen kombiniert. An vier Orten des Staates Sao Paulo, Brasilien, wurden Proben von 126 Bienenvölkern gesammelt. Zehn Individuen je Volk wurden nach den drei, bei Honigbienen polymorphen Enzymloci *Mdh*-1, *Pgm*-1 und *Hex* charakterisiert. Die morphometrische Analyse von 10 Bienen je Volk wurde nach dem Verfahren von Daly und Balling (1978) durchgeführt. Die mitochondriale DNA von allen Völkern wurde durch Abbauprodukte von Extrakten der Gesamtnukleinsäuren mit dem ECOR1- und BCL-1-Restriktionsenzym und Agarosegel-Elektrophorese charakterisiert. Die Restriktionsfragmente wurden durch Hybridisierung mit einer nick-translatierten Sonde hergestellt aus gereinigter Honigbienen-mtDNA, und nachfolgender Autoradiographie sichtbar gemacht. Als Gruppe waren die vier Bienenpopulationen nach ihrer Morphologie und der bei allen Individuen gezeigten "afrikanischen" mtDNA vollkommen 'afrikanisiert'. Aber die Allozymdaten von diesen Populationen stimmten mit früheren Untersuchungen überein, welche gezeigt haben, daß die brasilianischen afrikanisierten Bienen Abkömmlinge sowohl von afrikanischen wie von europäischen Vorfahren sind (Del Lama *et al,* 1990; Lobo *et al,* 1989; Moritz and Meusel, 1991). Die Homogenität der Schätzungen von Allozymhäufigkeiten bei den hier und früher untersuchten Populationen weist darauf hin, daß die Populationen aus dieser Region in ihrem genetischen Aufbau relativ stabil sind. Die morphologischen und mtDNA-Daten dieser neotropischen Populationen zeigen den starken Einfluß der afrikanischen Rasse *A m scutellata.* Die offensichtlichen Unstimmigkeiten zwischen den Ergebnissen dieser verschiedenartigen Untersuchungsmethoden geben unterschiedliche Einflüsse der Selektion und der Populationsgröße auf die Vererbung und den Bestand dieser Merkmale wieder und unterstreichen die Bedeutung einer Analyse nach unterschiedlichen Methoden.

afrikanisierte Honigbiene / Brasilien / Populationsgenetik / Morphometrie / mtDNA / enzymatischer Polymorphismus

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