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Original article

Identification of Africanized honey bees through wing morphometrics: two fast and efficient procedures*

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Abstract – Currently available morphometric and genetic techniques that can accurately identify Africanized honey bees are both costly and time consuming. We tested two new morphometric techniques (ABIS – Automatic Bee Identification System and geometric morphometrics analysis) on samples consisting of digital images of five worker forewings per colony. These were collected from 394 colonies of Africanized bees from all over Brazil and from colonies of African bees, *Apis mellifera scutellata* (n = 14), and European bees, *A. m. ligustica* (n = 10), *A. m. mellifera* (n = 15), and *A. m. carnica* (n=15) from the Ruttner collection in Oberursel, Germany (preserved specimens). Both methods required less than five minutes per sample, giving more than 99% correct identifications. There was just one misidentification (based on geometric morphometrics analysis) of Africanized bees compared with European subspecies, which would be the principal concern in newly-colonized areas, such as the southern USA. These new techniques are inexpensive, fast and precise.

Africanized honey bee / morphometrics / geometric morphometrics analysis / ABIS / *Apis mellifera* / automatic identification

1. INTRODUCTION

After the introduced African honey bee, *Apis mellifera scutellata* Lepeletier, escaped from a university apiary in Brazil in 1957, it crossed with the previously-established European bee races (Kerr, 1967); the resultant polyhybrid, named the 'Africanized bee' (Gonçalves, 1974), spread throughout most of South and Central America by 1987. Subsequently, it invaded Mexico and reached the USA in 1991 (Rinderer et al., 1993). Though the behavior of Africanized bees is considerably different from that of other European *Apis mellifera* races, they are quite similar in appearance; often beekeepers and the public only became aware of their presence after stinging incidents. Consequently, there has been considerable interest in methods for identifying Africanized honey bees so that proper management decisions can be made.

Various methods have been developed to identify Africanized bees, including analyses of isozymes (Contel et al., 1977; Del Lama et al., 1988), mitochondrial DNA polymorphisms (Hall and Muralidharan, 1989; Smith et al., 1989; Sheppard et al., 1991a, b; Segura, 2000), cuticular hydrocarbons (Francis et al., 1985), and nuclear DNA (Hall, 1988; Clarke et al., 2002; Whitfield et al., 2006). However, these biochemical and molecular methods

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require expensive reagents and laboratory equipment.

Morphometrics has been and continue to be the most widely-used official methodology for identifying Africanized honey bees, because of high practicability and low costs. The first effective procedure, developed in the 1970s, consisted of manually measuring 25 characters of the wings, sternites and legs with an ocular micrometer; these measures were then analyzed with multivariate statistics to make the identifications (Daly and Baling, 1978). This identification method was later improved by incorporation of computer-assisted measurements (Daly et al., 1982). A simplification of these procedures resulted in the "Fast Africanized Bee Identification System" (FABIS) for preliminary identification in the field (Rinderer et al., 1986). Whenever colonies are suspected to be Africanized based on FABIS, official identification in the USA is currently made with the USDA-ID (United States Department of Agriculture identification) method (Rinderer et al., 1993). This method uses 25 morphometric measurements, manually entered into a computer program, of five balsam-mounted parts of each bee (fore- and hindwing, femur-tibia, basitarsus of a hind leg and a sternite); though it has good precision, it requires skilled personnel and several hours of preparation and analysis per 10-bee sample. These limitations and continuing reductions in the costs of molecular biology technology have turned DNA recognition systems into an attractive alternative to morphometrics.

However, recent advances in statistical analysis and image recognition software have made morphometric analysis more precise and practical for identifying species. Schröder et al. (1995) developed a semi-automatic system for bee species recognition, based on characteristics extracted from the forewings; this system was named ABIS (Automatic Bee Identification System). The landmarks of this system were manually-plotted wing-vein junctions; after training the system with at least 30 individuals per group, the identification of individuals was quite fast. Several improvements were subsequently made to the original ABIS system; the new version requires little user input and has greater precision than the original version (Steinhage et al., 2001). ABIS is a supervised classification method; i.e. the program must be trained with at least 20 specimens of each class (such as species, race, or population). Currently, ABIS is able to automatically identify and mark the landmarks in the digital images of the wings. Another improvement is the use of non-linear discriminant analysis based on kernel functions (Roth and Steinhage, 1999), instead of linear discriminant analysis, for the differentiation of the groups. ABIS was able to discriminate European members of the genera *Colletes, Andrena* and *Bombus* (Steinhage et al., 2001) to the species level with a precision of 99.8%, and it correctly identified 94% of the bees in a comparison of honey bee subspecies (Drauschke et al., 2007; Francoy et al., in press).

Another modern morphometric method that is also very promising for shape studies is geometrics morphometrics based on the description of shape in Cartesian coordinates (Bookstein, 1991). Though they are very useful for reconstructing shapes, since the relative position of landmarks is retained during analysis (Rohlf and Marcus, 1993), the use of these techniques in honey bees has till now been restricted to analysis of fluctuating asymmetry in studies of hybridization (Smith et al., 1997; Schneider et al., 2003). We have found that this technique can be used to identify honey bee subspecies; in a preliminary study, we were able to identify 85% of the individuals in a comparison of European and Africanized bees (Francoy et al., in press).

Based on these initial studies, we investigated the utility of these two morphometric techniques (ABIS and geometric morphometrics analysis) for distinguishing Africanized honey bees collected from all regions of Brazil from the African and European subspecies that gave origin to this polyhybrid.

2. MATERIAL AND METHODS

Worker bees from 394 colonies of Africanized honey bees were collected from 24 different localities from 16 of the 24 states of Brazil (Tab. I).

Table I. Localities (city and state) and number of Africanized honey bee colonies sampled in Brazil (in 1997). N = number of colonies sampled.

Locality	Latitude	Longitude	N
Belém, PA	1° 26'S	48° 29'W	20
São Luis, MA	2° 31'S	44° 18'W	20
Fortaleza, CE	3° 46'S	38° 35'W	40
Baraúna, RN	5° 05'S	37° 36'W	6
Serra do Mel, RN	5° 10'S	37° 02'W	8
Mossoró, RN	5° 11'S	37° 20'W	5
Severiano Melo, RN	5° 46'S	37° 57'W	5
Catolé do Rocha, PB	6° 20'S	37° 45'W	3
Picos, PI	7° 05'S	41° 46'W	25
Crato, CE	7° 14'S	39° 25'W	22
Araripina, PE	7° 33'S	40° 34'W	20
Aracaju, SE	10° 54'S	37° 03'W	20
Tucano, BA	10° 58'S	38°47'W	20
Salvador, BA	12° 58'S	38° 30'W	13
Aquidauana, MS	20° 28'S	55° 47'W	19
Ribeirão Preto, SP	21° 10'S	47° 51'W	18
São João da Boa Vista, SP	21° 59'S	46° 47'W	12
Rio de Janeiro, RJ	22° 54'S	43° 12'W	20
Maringá, PR	23° 24'S	51° 55'W	10
Curitiba, PR	25° 25'S	49° 17'W	20
Florianópolis, SC	27° 35'S	48° 32'W	19
Santa Maria, RS	29° 41'S	53° 49'W	20
Alegrete, RS	29° 47'S	55° 47'W	14
Porto Alegre, RS	30° 02'S	51° 13'W	15

The Ribeirão Preto sample was collected in 2002.

Additionally, wings of workers from 14 colonies of *A. m. scutellata*, 10 colonies of *A. m. ligustica* Spinola,15 colonies of *A. m. mellifera* L. and 15 colonies of *A. m. carnica* Pollmann were obtained from the Morphometric Bee Data Bank in Oberursel, Germany (the Ruttner collection) and used as parameters of pure subspecies in the comparisons. These four subspecies were selected because they were the main subspecies introduced into Brazil (Francoy et al., in press).

The right forewings of approximately five bees per colony were mounted between microscope slides and photographed with a digital camera attached to a stereomicroscope. The wings of the subspecies from the Ruttner collection were also photographed with the same equipment and to the same scale.

We analyzed the bee wings individually with ABIS (Steinhage et al., 2001). Initially, ABIS performs a detailed analysis of the venation of the wings. The geometry and structure of this venation network is used by this software as fingerprints for bee species (Steinhage et al., 2001). The measures made by ABIS are described in Drauschke et al. (2007). In a second step, a statistical classification is made using nonlinear kernel discriminant analysis (Roth and Steinhage, 1999).

For the geometric morphometrics analysis, 19 homologous landmarks were manually plotted at the wing vein intersections (Fig. 1) using the software tpsDig, version 2.04 (Rohlf, 2005); these images were then Procrustes aligned (Bookstein, 1991). After alignment of the Cartesian coordinates of the wings, the mean configuration of the bees from a colony was used as a comparative parameter and the identifications were made at the colony level. A forward stepwise analysis (tolerance 0.01; F to enter 1.00) was carried out to determine classification functions, followed by a canonical analysis and then a cross validation test to check the accuracy of the equations in identifying the colonies.

3. RESULTS

3.1. ABIS

We ran a cross validation test 30 times for all the groups; each time 10% of the individuals (randomly selected) were used as unknowns (Tab. II). During this test, the nonlinear Kernel Discriminant Analysis (KDA) of ABIS correctly identified 98.05% of the individuals from all the groups. If we take into consideration just the Africanized bee specimens, among 5280 identifications, only four Africanized bees were incorrectly identified as *A. m. scutellata* (0.08%). The most common errors in the identifications made with ABIS were for the subspecies *A. m. ligustica* and *A. m. carnica.* ABIS was able to correctly identify 80% of the *A. m. ligustica* and 85% of the *A. m. carnica* samples.

3.2. Geometric morphometrics analysis

Among the 38 Cartesian coordinates, 31 were used in the classification model of the five groups. Using multivariate analysis of variance (MANOVA), we found significant differences between the groups (Wilk's λ = 0.03623; $P < 0.0001$). The Mahalanobis

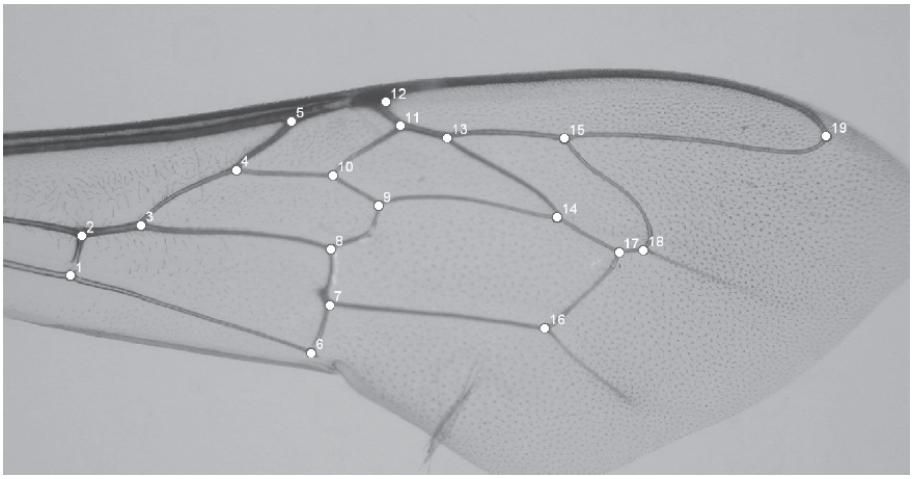

Figure 1. Forewing of Africanized honey bee worker with 19 landmarks plotted in the vein junctions.

square distances between the centroids were also significant (linear discriminant analysis, $P < 0.00001$).

Cross validation tests based on linear discriminant functions correctly identified 97.8% of the colonies (Tab. III). The mean configuration of the Cartesian coordinates and the discriminant functions for each group are presented in additional online material (Appendix I and II, respectively). Among the Africanized bee samples, 99.2% of the colonies were correctly identified; 0.5% of the Africanized colonies (n = 2) were identified as being *A. m. scutellata* and just one was wrongly identified as *A. m. ligustica.* The most common error was misidentification of the *A. m. scutellata* samples: 50% of the *A. m. scutellata* colony samples were incorrectly identified as Africanized honey bees.

4. DISCUSSION

Though molecular methods work well for identifying Africanized bees (Hall, 1988; Hall and Muralidharan, 1989; Smith et al., 1989; Sheppard et al., 1991a; Segura, 2000; Clarke et al., 2002; Whitfield et al., 2006), they are expensive, require specially-trained personnel and are time consuming. Morphometric identification techniques, which have improved

Table II. Identifications of *Apis mellifera* subspecies based on ABIS (Steinhage et al., 2001) morphometric analysis of forewings. Identifications were made of each bee. There were five bees per colony sample. The first column indicates the expected classifications (based on previously-determined sample identity); N = the number of colony samples; the numbers under the following columns show the observed classifications.

		Identification based on ABIS (%)				
Expected classification	N	AHB	*A. m. ligustica*	*A. m. scutellata*	*A. m. mellifera*	*A. m. carnica*
AHB	394	99.92	0.00	0.08	0.00	0.00
A. m. ligustica	10	1.25	80.00	2.50	0.00	16.25
A. m. scutellata	14	3.85	0.26	94.10	1.79	0.00
A. m. mellifera	15	0.00	1.67	2.50	95.00	0.83
A. m. carnica	15	2.08	11.25	0.83	0.00	85.83

AHB, Africanized honey bee.

Table III. Identification of *Apis mellifera* colony samples based on geometric morphometrics analysis of five forewings per colony. The first column indicates the known classifications (previously-determined sample identity), N = number of colonies; the numbers under the following columns show the classifications based on the morphometric analysis.

		Identification based on geometric morphometrics analysis (%)				
Known classification	N	*A. m. carnica*	*A. m. ligustica*	*A. m. mellifera*	*A. m. scutellata*	AHB
A. m. carnica	15	93	7	0	0	0
A. m. ligustica	10	0	100	0	0	0
A. m. mellifera	15	0	0	100	0	0
A. m. scutellata	14	0	0	0	50	50
AHB	394	0	0.3	0	0.5	99.2

AHB, Africanized honey bee.

considerably due to new computational techniques, are currently more practical since they require little technical knowledge or specialized equipment.

With the evolution of computational morphometrics identification systems, it is now possible to identify species of various groups of insects using only wing features (Steinhage et al., 2001; Tofilski, 2004; Francoy et al., 2006; Steinhage et al., 2007; Drauschke et al., 2007; Mendes et al., 2007). Here, we have extended this strategy to a large sample of Africanized honey bees. There were few misidentifications with these two methods; just one of the Africanized bee colonies was incorrectly identified (based on geometric morphometrics analysis) as being bees of European subspecies, which normally would be the main concern for such analyses. This high level of precision, along with the rapidity of the technique, and the relatively low cost of the necessary infrastructure (stereomicroscope, digital camera, computer and software), makes these two methods attractive alternatives for routine identifications. Once ABIS is trained with a data set of bees, the identification of each individual takes no longer than two minutes; the same procedure with geometric morphometrics analysis of colonies takes about five minutes. All the softwares that we used for geometric morphometrics analysis are freely available on the internet at http://life.bio.sunysb.edu/morph/. Though it is not available to the general public, the ABIS software can be used in cooperation with the Institute of Informatics III at the University of Bonn.

Very few samples of Africanized honey bees were incorrectly classified as pure *A. m. scutellata*; which is no longer found in the Neotropics. Rinderer et al. (1990) also concluded that the AHB has a morphometric profile different from that of *A. m. scutellata*. Here we add that it is also different from the three European subspecies that had been previously introduced to Brazil.

Currently, classic morphometric identification methodologies for Africanized honey bees, such as USDA-AID (Rinderer et al., 1993), are time consuming. Both of these new methodologies, ABIS and geometric morphometrics analysis, proved to be very efficient and fast for the identification of Africanized honey bees, and thus would be useful for such identifications in new or recently-colonized areas and for border control programs.

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Deux procédés rapides et efficaces pour identifier les abeilles africanisées à l'aide de la morphométrie de l'aile.

Apis mellifera **/ abeille africanisée / morphométrie / ABIS / identification automatique**

Zusammenfassung – Zwei schnelle und effiziente Verfahren zur Identifikation Afrikanisierter Honigbienen anhand der Flügelmorphometrie. Die Afrikanisierten Honigbienen sind unter den verschiedenen Unterarten und Rassengruppen der Honigbiene (*Apis mellifera* L.) in den Neotropen und den Nachbarregionen am meisten respektiert und gefürchtet, insbesondere da sie in neue Gebiete einwandern. Die Identifizierung der Afrikanisierten Bienen ist in diesen Regionen für die Bewirtschaftung der Bienenvölker daher unverzichtbar. Sie ermöglicht die Bestimmung ihres Verbreitungsgebiets und ihrer Ausbreitungsgeschwindigkeit, dies ist sowohl für die Imker als auch für die damit befassten Regierungseinrichtungen von Bedeutung.

Wir benutzten zwei kürzlich entwickelte morphometrische Techniken (ABIS – Automatic Bee Identification System und die Geometrische Morphometrische Analyse), um Proben aus jeweils fünf rechten Vorderflügeln pro Volk zu analysieren (Tab. I). Beide dieser Methoden benötigten in einem Vergleich von 394 über ganz Brasilien verteilten Völkern weniger als 5 Minuten pro Volk und erreichten eine mehr als 99% korrekte Identifizierung. Diese ergaben 14 Völker von *A. m. scutellata*, 10 Völker von *A. m. ligustica*, 15 Völker von *A. m. mellifera* und 15 Völker von *A. m. carnica* (Tab. II und III). Mit ABIS können einzelne Bienen bestimmt werden, während die Geometrische Morphometrische Analyse eine auf jeweils 5 Flügeln beruhende Identifikationen auf Kolonieebene durchführt. Die meisten der Fehleinordnungen fanden zwischen Afrikanisierten und Afrikanischen Bienen sowie zwischen den europäischen Unterarten statt. Nur eines der Afrikanisierten Bienenvölker wurde irrtümlich als eine europäische Unterart eingeordnet, dies ist die Fehlerart die insbesondere innerhalb von neubesiedelten Gebieten wie den Südstaaten der USA von Bedeutung wäre. Die erreichten Fortschritte in Computertechnologie, statistischen Analysen und Bilderkennungssoftware sowie die verbesserten Informationen über die relevanten Messgrößenbereiche und die höhere Genauigkeit und größere Geschwindigkeit der Messungen selbst machen es nun möglich, Afrikanisierte Bienen ausschließlich anhand von Digitalaufnahmen der Vorderflügel in Minutenschnelle zu identifizieren.

Afrikanisierte Honigbienen / Morphometrie / Geometrische Morphometrische Analyse / ABIS / *Apis mellifera* / automatische Identifikation

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Online Material

Appendix I. Mean values of the Cartesian coordinates according to the respective group and the mean configuration of all the colonies.

	AHB	*A. m. scutellata*	*A. m. mellifera*	*A. m. ligustica*	*A. m. carnica*	all
1x	-0.3448	-0.3487	-0.3467	-0.3469	-0.3476	-0.3451
1y	-0.1296	-0.1313	-0.1303	-0.1317	-0.1305	-0.1297
2x	-0.3411	-0.3441	-0.3446	-0.3430	-0.3445	-0.3414
2y	-0.0833	-0.0837	-0.0839	-0.0834	-0.0820	-0.0833
3x	-0.2881	-0.2874	-0.2930	-0.2943	-0.2933	-0.2885
3y	-0.0639	-0.0637	-0.0652	-0.0663	-0.0646	-0.0640
4x	-0.1823	-0.1786	-0.1811	-0.1829	-0.1835	-0.1822
4y	0.0179	0.0190	0.0180	0.0212	0.0207	0.0181
5x	-0.1327	-0.1321	-0.1364	-0.1378	-0.1386	-0.1332
5y	0.0776	0.0768	0.0748	0.0786	0.0782	0.0775
6x	-0.0696	-0.0710	-0.0673	-0.0623	-0.0625	-0.0692
6y	-0.1713	-0.1719	-0.1697	-0.1742	-0.1735	-0.1714
7x	-0.0559	-0.0564	-0.0520	-0.0507	-0.0521	-0.0555
7y	-0.1168	-0.1168	-0.1174	-0.1193	-0.1176	-0.1169
8x	-0.0611	-0.0599	-0.0595	-0.0555	-0.0559	-0.0607
8y	-0.0541	-0.0549	-0.0529	-0.0533	-0.0535	-0.0541
9x	-0.0134	-0.0119	-0.0110	-0.0020	-0.0035	-0.0127
9y	0.0065	0.0063	0.0098	0.0082	0.0083	0.0067
10x	-0.0636	-0.0620	-0.0614	-0.0603	-0.0571	-0.0632
10y	0.0278	0.0281	0.0280	0.0323	0.0316	0.0280
11x	-0.0070	-0.0051	-0.0071	-0.0066	-0.0068	-0.0069
11y	0.0917	0.0922	0.0901	0.0942	0.0930	0.0918
12x	-0.0298	-0.0289	-0.0294	-0.0292	-0.0305	-0.0298
12y	0.1141	0.1146	0.1136	0.1177	0.1163	0.1142
13x	0.0502	0.0502	0.0523	0.0498	0.0508	0.0503
13y	0.0895	0.0894	0.0872	0.0926	0.0913	0.0895
14x	0.1818	0.1795	0.1834	0.1765	0.1770	0.1815
14y	0.0239	0.0248	0.0245	0.0236	0.0223	0.0239
15x	0.1730	0.1739	0.1741	0.1593	0.1561	0.1722
15y	0.1085	0.1079	0.1067	0.1072	0.1044	0.1083
16x	0.1876	0.1887	0.1848	0.1904	0.1936	0.1878
16y	-0.1030	-0.1022	-0.0981	-0.1041	-0.1028	-0.1028
17x	0.2553	0.2556	0.2516	0.2556	0.2594	0.2553
17y	-0.0018	-0.0014	0.0015	-0.0019	-0.0025	-0.0017
18x	0.2861	0.2861	0.2889	0.2859	0.2874	0.2862
18y	0.0061	0.0076	0.0102	0.0077	0.0065	0.0063
19x	0.4554	0.4522	0.4545	0.4542	0.4517	0.4551
19y	0.1603	0.1592	0.1530	0.1509	0.1543	0.1596

Appendix II. Classification functions for the discrimination of the Africanized honey bees and the subspecies that were compared with the Africanized bees. The functions were calculated based on the Cartesian coordinates (CC) of the vein junctions.

	A. m. carnica	*A. m. ligustica*	*A. m. mellifera*	*A. m. scutellata*	AHB
9X	-165025	-164154	-162947	-166132	-165481
19Y	1189204	1185782	1182402	1188857	1186436
15X	-2448	-1661	420	-1507	-584
18Y	808265	807988	803183	807527	804127
9Y	147610	146353	149461	146272	146419
10Y	484664	483617	478210	481943	481561
15Y	512855	513191	512930	514914	514174
13Y	574236	572514	565296	568592	568143
14Y	447740	447914	446987	452087	450421
14X	136993	137985	139978	138453	139227
11Y	337904	335977	337426	340595	338965
17X	470162	469789	469404	469596	469937
7Y	-167548	-169053	-170912	-168476	-169057
18X	281223	281305	283388	280467	281042
13X	-165991	-165292	-163850	-167050	-165661
4X	-531378	-530706	-529039	-531691	-531389
5X	-372768	-371736	-369818	-371517	-370564
6X	158357	157582	156126	156548	156419
7X	-155216	-154189	-152244	-154433	-154218
10X	-237738	-237645	-236994	-238598	-237818
12X	-263065	-261729	-259317	-262616	-261064
19X	349587	350525	351820	349482	350915
16Y	415144	412713	412288	414201	412724
17Y	490425	486815	486262	487060	486571
12Y	258842	258912	259112	258139	257599
1X	-462812	-463048	-461941	-463278	-462985
1Y	-452792	-452134	-450705	-453884	-452113
3Y	-820759	-820463	-817061	-820868	-819227
2X	-729386	-728650	-727609	-729386	-728715
11X	-266281	-265910	-264512	-265844	-265387
5Y	282716	281837	281725	281604	282362
Constant	-726214	-725814	-724850	-726443	-726240