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An improved test for Africanized honeybee mitochondrial DNA

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Abstract. Mitochondrial DNA derived from *Apis mellifera scutellata*, the ancestor of the Africanized bees of the New World, lacks a *Bgl*II restriction site found in other types of honeybee [1,2]. We present primers allowing amplification of a 485-bp section of the cytochrome b gene containing this site, using the polymerase chain reaction. Digestion of the amplified product with *Bgl*II yields contrasting patterns between Africanized and other honeybees.
Key words. Africanized honeybee; *Apis mellifera scutellata*; mitochondrial DNA; restriction fragment length polymorphism; *Bgl*II; polymerase chain reaction.

The introduction of *Apis mellifera scutellata* from Africa into the New World at São Paulo, with the aim of improving apiculture, led to the dramatic spread of these bees [3,4]. The genetical evidence so far supports the notion that the Africanized bees have spread almost wholly by ecological displacement of the commercial, European-derived strains, rather than by superior mating success, in that a strong association between nuclear and mtDNA RFLPs remains even in the northernmost populations of the advancing front [1,2,5]. It has been argued [1,5] that the introduced bees have interbred sufficiently little with other strains as to be termed African and not Africanized, but we prefer to retain the latter term in this paper.

As noted by Taylor and also McDowell (Hall [5]), Africanized bees are aggressive, difficult to manage, and liable to disrupt bee-based pollination and other agricultural activities. The economic impact of Africanized bees is therefore liable to spread considerably beyond the effects on honey production. A rapid, easy, and reliable test for Africanized bees would be valuable not only for testing reports of incursions of these bees into new areas, but also for continuing research into the evolutionary dynamics of the spread of this colonising insect. We report a test based on mtDNA and PCR which would permit easy identification of Africanized matrilines.

The restriction enzyme *Bgl*II cuts the mtDNA of Africanized bees into one and that of non-Africanized bees into two fragments [1,2]. *Eco*RI digestion patterns also discriminate between bees of different origins, but not as simply [1,2].

Published [6,7] and our unpublished sequence studies show that the rRNA genes and the genes encoding the proteins cytochrome oxidase subunits I, II, and III, cytochrome b, and ATPase subunits 6 and 8, are in the same relative positions as for *Drosophila yakuba* [8], but that there have been shifts in tRNA genes. We have therefore identified the position of the diagnostic *Bgl*II site within the cytochrome b gene (fig. 1) and designed appropriate primers to amplify a 485 bp portion of this gene containing the site.

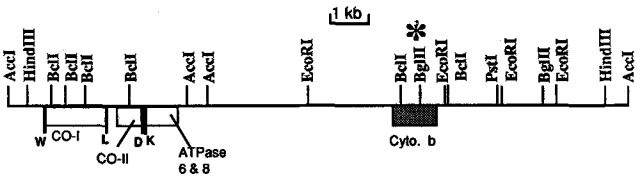

Figure 1. Restriction map of *Apis mellifera ligustica* mtDNA showing the location of the cytochrome b gene and of genes completely sequenced so far (CO-I and CO-II: genes for cytochrome c oxidases subunits 1 and 2, W, L, D, K: tRNA genes as denoted by the one-letter amino acid code). The asterisk indicates the *Bgl*II site whose absence characterises Africanized bees.

Materials and methods

Africanized and non-Africanized adult and larval bees were collected into 95% ethanol and sent to us by H. G. Hall [5] at ambient temperatures. Adult bees were dried to remove ethanol, while larval specimens were equilibrated overnight [1] before total DNA extraction using a modification of the CTAB method of Boyce et al. [9]. Individual bees were quick frozen in liquid N_2, ground using mortar and pestle and digested at 65 °C for 2 h in 500 µl of 2 × CTAB buffer (0.1 M Tris-HCl (pH 8.3), 0.02 M EDTA (pH 8), 1.4 M NaCl, 0.2% betamercap-

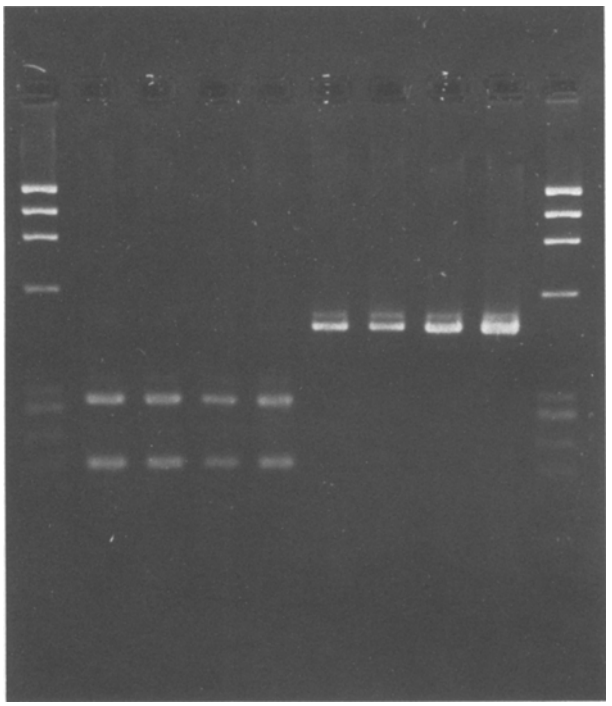

Figure 2. DNA amplified from individual bees, using the primers described, and cut with *Bgl*II. Lanes 1 and 10 contain size standards (ϕX-174 DNA cut with *Hae*III), lanes 2–5 are from non-Africanized bees (Hall's[5] codes BL-6, BL-8, BL-9, BL-10) and lanes 6–9 are from Africanized bees (Hall's[5] codes VCN-SW5, VCN-7A, AF-65W, AF-67W). All bees used were adults except for AF-65W and AF-67W.

toethanol (v:v), 0.05 M hexadecyltriethylammonium bromide). Debris was removed by microfuge centrifugation at 13,000 rpm for 10 min. The supernatant was extracted once with the addition of 500 µl phenol/chloroform/isoamyl alcohol (25:24:1) and twice with 500 µl chloroform/isoamyl alcohol (24:1) before overnight precipitation of DNA with 1.5 vols of isopropanol at −20 °C. The DNA was pelleted, washed twice with 100 µl 70% ethanol, dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). The polymerase chain reaction (PCR) was performed as described (Saiki[12]) but with 1 µl of DNA, 1 µl of a 25 µM stock of each primer and 2 units of Taq polymerase (Promega) in a 50-µl reaction volume. The reaction mix was initially denatured at 92 °C for 5 min before addition of enzyme and 35 cycles of annealing at 53 °C, extension at 70 °C and dissociation at 92 °C using an Inovonics thermal cycler (ending with a 2-min extension step). The sequences of the primers used were:

5′TATGTACTACCATGAGGACAAATATC and,
5′-ATTACACCTCCTAATTTATTAGGAAT.

Following amplification, 10 µl of 1 × restriction buffer was added to 5 µl of PCR product with 2–3 units of *Bgl*II (Boehringer) and digested for 3 h. The total digestion volume was loaded into a 1% NuSieve and 3% Seakem (FMC) agarose minigel, electrophoresed in TAE buffer and stained with ethidium bromide.

Results

The procedure results in two strongly contrasting patterns (fig. 2): one with the 485 bp PCR product cut into 291 bp (5′ to the *Bgl*II site on the coding strand) and 194 bp fragments by *Bgl*II (non-Africanized bees) and one in which it remains uncleaved (Africanized bees). These findings hold for all of 15 Africanized strains and 10 non-Africanized sent to us by H. G. Hall.

Discussion

MtDNA is maternally inherited in bees as in other metazoa generally (rare exceptions may occur in *Drosophila*[10]): although paternal mtDNA can make up to 10% of the total mtDNA in the fertilized egg, it is lost by the larval stage[11]. Hence there is no recombination and a single clear marker is sufficient to trace the lineage of the entire molecule. Hall and Smith (in press) report that the mtDNA of Africanized bees can be identified by the joint absence of an EcoRI site in the lsRNA gene and a *Hinc*II site within the CO-I gene using a rapid PCR-based procedure. The test described here, based on a single *Bgl*II site, should prove a rapid, easy, and reliable aid in the study of the molecular biology of this invading population. The procedure can be performed much more quickly and easily than a restriction digest of total DNA (which involves Southern blotting and hybridization against a suitable probe) or a restriction digest and end-labelling of CsCl-purified mtDNA, and requires much less tissue.

Note added in proof: The Africanized pattern detectable with our assay also occurs in bees from southern Spain (D. R. Smith et al., in press).

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