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Genetic Similarity among Male Bees of *Euglossa truncata* Rebêlo & Moure (Hymenoptera: Apidae) Revealed by RAPD Markers

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Similaridade Genética entre Machos de *Euglossa truncata* Rebêlo & Moure (Hymenoptera: Apidae) Revelada por Marcadores RAPD

RESUMO - Os marcadores genéticos RAPD (DNA polimórfico amplificado ao acaso) têm sido empregados com sucesso em estudos taxonômicos de diversos grupos de organismos. No presente trabalho esses marcadores moleculares foram utilizados para analisar a similaridade genética entre dezoito machos de *Euglossa truncata* Rebêlo & Moure apresentando variações em dois caracteres morfológicos (coloração do escapo antenal e da pilosidade dos tufos metaepisternais), os quais normalmente são utilizados na identificação dessa espécie de abelha Euglossina. Os 12 *primers* utilizados nas análises produziram 127 locos de RAPD, dos quais 40 (31,5%) foram polimórficos, revelando alguma variação genética entre os indivíduos analisados. Os coeficientes de similaridade genética entre os indivíduos variaram de 0,79 a 0,95, indicando alta similaridade entre os 18 machos analisados. Nenhuma banda de RAPD mostrou-se específica para os caracteres morfológicos considerados. Os resultados obtidos indicam que todos os machos analisados pertencem à mesma espécie euglossina. A alta similaridade genética entre os dezoito machos eulossíneos indica que as variações observadas nos caracteres morfológicos destes não estão em discordância com a identificação dessa espécie de Euglossina, e que tais caracteres podem variar entre os machos de *E. truncata*.

PALAVRAS-CHAVE: Apoidea, Euglossini, abelha das orquídeas, abelha euglossina, marcador molecular

ABSTRACT - The genetic RAPD (random amplified polymorphic DNA) markers have been used successfully in taxonomical studies of several groups of organisms. In the present study these molecular markers were used to analyze the genetic similarity among eighteen males of *Euglossa truncata* Rebêlo & Moure exhibiting variations in two morphological characters (colour of the antennal scape and metaepisternal hairs) which are frequently used to identify this species of euglossine bee. The twelve primers used in the RAPD analysis amplified 127 loci, of which 40 (31.5%) were polymorphic, showing some variation among the individuals. The coefficients of genetic similarity among the individuals ranged from 0.79 to 0.95, indicating a rather high genetic similarity among the 18 male bees studied. No RAPD band was specific to any morphological character analyzed. The results indicate that all bees analyzed belong to the same species. The high genetic similarity among the eighteen euglossine males studied indicates that the variations observed in the morphological characters are not in disagreement with the identification of this species of Euglossina and these characters can vary among males of *E. truncata*.

KEY WORDS: Apoidea, Euglossini, orchid bee, euglossine bee, molecular marker

The genus *Euglossa* Latreille is the largest of subtribe Euglossina (Hymenoptera: Apidae, Apinae, Apini), with more than a hundred species of bees (Cameron 2004, Roubik & Hanson 2004), many of them described in recent times (Moure 1995, Rebêlo & Moure 1995, Moure & Schlindwein 2002). The genus includes small to medium-large bees (8-18mm long), with relatively sparse hairs and shiny metallic integument, usually green, blue or bronze (Dressler 1982a). The males have conspicuous white marks about the mouthparts, which are smaller and less obvious in females;

the male hind tibia is broad, more or less rhomboid or triangular and rounded distally (Dressler 1982a). Actually, most of structural characteristics used to distinguished euglossine genera from each other, and these bees from their relatives, are secondary sexual modifications of the males; genitalic structures as well as those involved in the collection and storage of aromatic chemicals (Kimsey 1980, 1987). Currently, *Euglossa* is subdivided into six subgenera and 12 species groups (Cameron 2004, Roubik & Hanson 2004).

In 1995, Rebêlo & Moure published a helpful dichotomous key for the identification of eleven species of *Euglossa* found in northeast of São Paulo State, southeast Brazil. In the same study, these authors described four new species of this genus, including the species *Euglossa truncata* Rebêlo & Moure, which belongs to the subgenus *Euglossa*, the largest of the genus (Cameron 2004). However, two morphological characteristics – antennal scape back and the hairs of metaespisternal tuft predominantly black – used to identify *E. truncata* in the key proposed by Rebêlo & Moure (1995), frequently vary in male bees collected in northern of Paraná State (neighboring São Paulo State), sometimes rendering identification of these specimens uncertain.

In the last few decades, PCR-based DNA markers, such as those generated by random amplified polymorphic DNA, RAPD (Welsh & McClelland 1990, Williams *et al.* 1990), have been found effective to distinguish among subspecies of various species of bee (Suazo *et al.* 1998, Waldschmidt *et al.* 2000, Oliveira *et al.* 2004). RAPD has been described as a simple and fast method of detecting polymorphism based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence, which requires minimal amounts of DNA and no previous knowledge of the genome (Welsh & McClelland 1990, Williams *et al.* 1990). Sources of polymorphism detected through of RAPD assays are normally represented by base substitutions within either priming site or deletions and insertions in priming sites (Williams *et al.* 1990, Dowling *et al.* 1996), which affect the efficiency of amplification, changing the profile of fragments produced by a given primer.

When RAPD markers are applied at appropriate taxonomic level, they can bee a powerful tool for studies of the genetics, systematics, and ecology of populations (Dowling *et al.* 1996). By far the most common use of RAPDs has been to identify and discriminate among individuals, cultivars, varieties, subspecies and species (Mailer *et al.* 1994, Fungaro *et al.* 1996, Nigatu 2000, Waldschmidt *et al.* 2000, Ghany & Zaki 2003).

The present study made use of RAPD markers to compare euglossine males previously identified as members of the species *E. truncata*, showing morphological variation, to confirm whether they really belonged to a single species.

Material and Methods

Study sites. The male euglossine bees were sampled in two green areas, away from each other 3.5 km, within the city of Londrina (23°18'37''S; 51°09'46''W), Paraná State, southern Brazil. The sites were denoted AT (Parque Arthur Thomas; 85.5 ha) and AF (wood area bordering Córrego Água Fresca; approx. 15 ha).

Bees were captured with an insect net when they were attracted to chemical baits of eucalyptol and eugenol (Sofia & Suzuki 2004). A total of 22 bees were collected: 13 from AF and 9 from AT. From these, four individuals, showing morphological differences, were kept as voucher specimens and 18 (8 from AT and 10 from AF) were used in RAPD analysis (Table 1).

Once caught, the bees were placed in plastic tubes, transported live to the laboratory and kept frozen (-20ºC) until the extraction of DNA. Bees were identified by one of the authors (S.H. Sofia) and voucher specimens are deposited at the Museu de Zoologia of the Univerisidade Estadual de Londrina (MZUEL).

Total genomic DNA was extracted from the 18 bees by procedures adapted from Raeder & Broda (1985). Before extraction, each bee was rinsed in a sterilized plate with distilled water, to remove from the insect integument any kind of DNA-containing contaminants (e.g. grains of pollen or fungal spores). After that, the thorax and abdomen of the bee were ground under liquid nitrogen to a fine powder, with a mortar and pestle. This material was subsequently homogenized in a microcentrifuge tube with 700 µl of extraction buffer (1% SDS - sodium dodecyl sulfate, 200 mM Tris-HCl, 250 mM NaCl, 25mM EDTA pH 8.0), with 5 µl of proteinase K (20 $\mu g.\mu l^{-1}$) in each tube. Next, the samples were incubated for 2h at 64°C, and then genomic DNA was extracted, with an equal volume of: phenol, (PCI) phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) homogenized and centrifuged. The upper (aqueous) layer was extracted with chloroform/isoamyl alcohol (24:1, v:v) homogenized and centrifuged at 6100 *g* for 10 min. The DNA in the aqueous layer was precipitated with two volumes of ice-cold ethanol and 10% by volume of 3 M NaCl. Finally, DNA was pelleted at 10400 *g* for 15 min, washed with 100µl of 70% ethanol, dried at room temperature and resuspended in 100µl of TE (10 mM Tris, 1 mM EDTA pH 8.0). Concentration was determined in a DyNA Quant 200 fluorometer (Hoefer), using the dye Hoechst 33258, using the dye Hoechst 33258, and diluted to a standard DNA concentration of $5ng.\mu l^{-1}$. All DNA-samples were then either used immediately or stored at -20°C.

The RAPD profiles were generated from total genomic DNA as described by Williams *et al.* (1990). Amplification reactions were performed in a total volume of 15 µl containing 15 ng of template DNA, 250 µM dNTP (Pharmacia), 0.25 µM of ten-nucleotide primer (Operon Technologies, Alameda, CA, USA), 4.5 mM $MgCl_2$ and 1 U of DNA polymerase in the reaction buffer supplied. The RAPD technique is sensitive to changes in reaction conditions (e.g. primer, $MgCl_2$, dNTP concentrations, temperature etc), so exactly the same reaction conditions were used for all samples. For the RAPD analysis, 39 different decamer oligonucleotide primers (kits OPX and OPAM) were tested and 12 were selected, that produced a good number of amplified bands and patterns of reproducible fragments. Control reactions were run with all components except genomic DNA and none of the primers used yielded

Table 1. Morphological characters of each of the 18 males of *E. truncata* collected in Parque Arthur Thomas (AT) and in a wooded area around Córrego Água Fresca (AF) in Londrina city, Brazil.

Bee	Area	Antennal scape	Tuft of metaepisternal hairs
Eg1	AT	Entirely black	Black
Eg2	AT	Entirely black	Black and white
Eg3	AF	Entirely black	Black
Eg4	AF	Entirely black	Black and white
Eg5	AF	Entirely black	Black
Eg6	AF	Black with a little white mark	White
Eg7	AT	Black with a little white mark	Black
Eg8	AT	Black with a little white mark	Black
Eg9	AT	Black with a little white mark	Black
Eg10	AF	Black with a little white mark	Black and white
Eg11	AF	Black with a little white mark	Black
Eg12	AT	Black with a large white mark	Black
Eg13	AT	Black with a large white mark	Black and white
Eg14	AT	Black with a large white mark	Black
Eg15	AF	Black with a large white mark	Black
Eg16	AF	Black with a large white mark	Black
Eg17	AF	Black with a large white mark	Black and white
Eg18	AF	Black with a large white mark	Black

detectable amplified products in these reactions. DNA amplifications were carried out in a thermal cycler (MJ Research PTC-100) and the amplification protocol consisted of 4 min at 92°C followed by 40 cycles of 40 s at 92°C, 1.5 min at 40°C, and 2 min at 72°C. The last round of amplification was followed by an additional extension at 72°C for 5 min.

Samples of 15 µl of amplification products were assayed by electrophoresis run at 3 $V.cm^{-1}$ in 1.4% agarose gels with TBE buffer (0.89 mM Tris, 0.89 mM boric acid, 2 mM EDTA, pH 8.3) diluted 1:20 (v:v). Gels were then stained with ethidium bromide, photographed under UV light using T-Max 100 Kodak film and scored visually for band presence and absence.

Comparative analyses were carried out by placing all samples on the same gel. Bees were arranged on the gel according to their morphological characters. The RAPD marker profiles were determined by direct comparison of the amplified DNA electrophoresis profiles, and each band was analyzed as a binary variable (present or absent). Only RAPD bands that were scored unequivocally were counted in the analysis.

To determine the similarities among individuals, a similarity matrix was constructed from the Jaccard coefficients of similarity (J), and then a UPGMA cluster based on J values was generated using the NTSYS-PC package (Rohlf 1992). To evaluate the robustness of the groupings formed the bootstrap analysis, with 1,000 replications, was performed using the Bood software program (Coelho 2000).

Results and Discussion

In Brazil, the species *E. truncata* has been found throughout the country, from north to south, in the States of Maranhão (Silva & Rebêlo 1999), Bahia (Neves & Viana 2003), Minas Gerais (Nemésio 2003), São Paulo (Rebêlo & Moure 1995) and Paraná (Sofia & Suzuki 2004, Sofia *et al.* 2004). According to Rebêlo & Moure (1995), males of this species characteristically show a violet clypeus, antennal scape totally black, tuft of metaepisternal hairs predominantly black and a truncated scutellum. The morphological characteristics of the 18 males of *E. truncata* used in the RAPD analysis in this study are listed in Table 1.

The twelve selected primers amplified a total of 127 reproducible RAPD fragments from the DNA isolated from the 18 male bees analyzed. Out of these 127 fragments, 40 (31.5%) were polymorphic, indicating some genetic variation among the euglossine males studied. The number of fragments per primer ranged from 7 to 16. The greatest number of bands was amplified with primer OPX6 (Fig. 1a). In Fig. 1 are shown several monomorphic bands shared by all males analyzed, as well as, two polymorphic bands

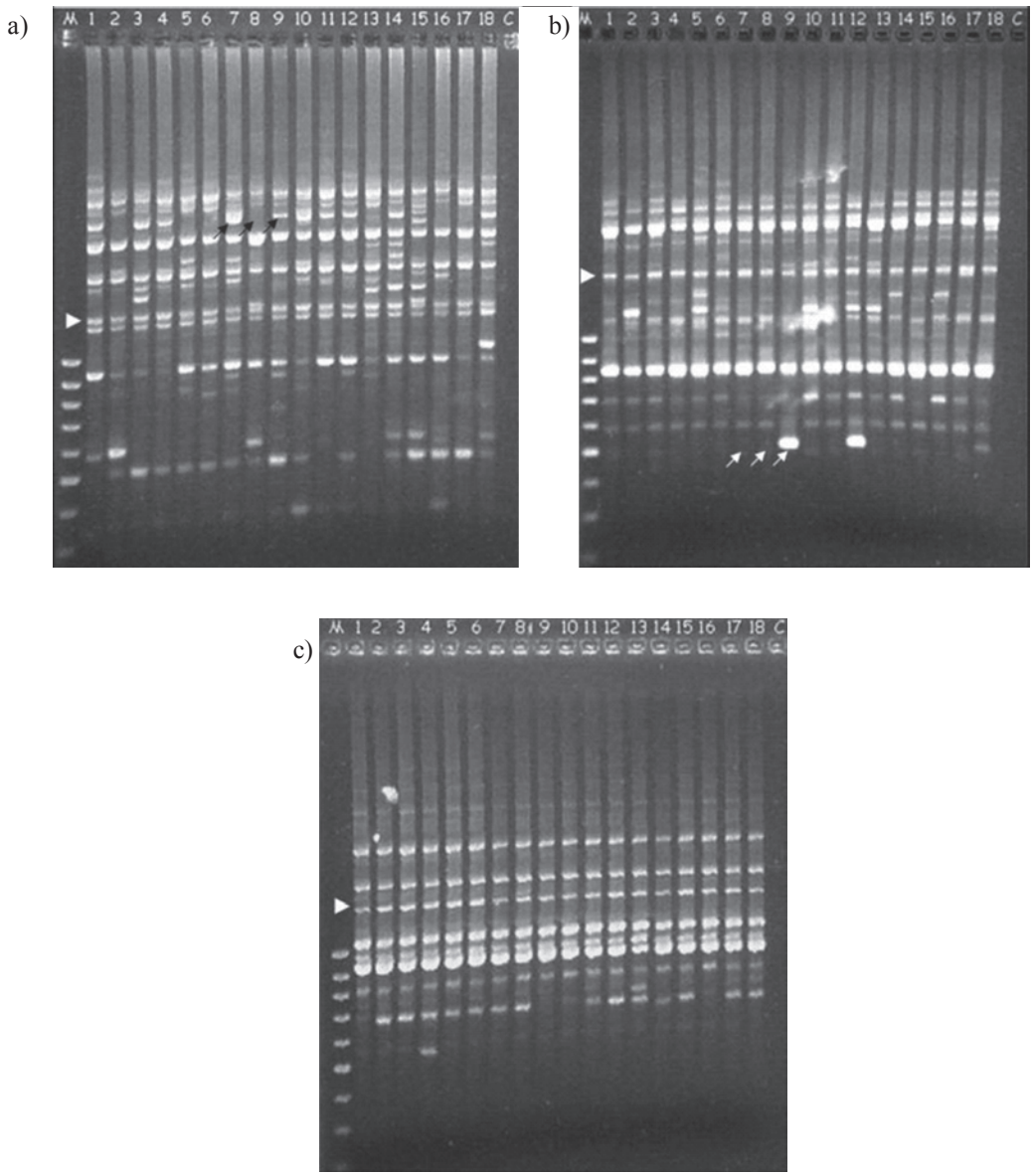

Figure 1. DNA polymorphism of the 18 males of *E. truncata* from Parque Arthur Thomas and in a wooded area around Córrego Água Fresca, located in Londrina city, amplified with primers: a) OPX6, b) OPX4 and c) OPX16. Column M = 100bp molecular weight marker (Biotools). Bees were arranged according to their morphology and the numbers (1 to 18) correspond to the bees (Eg1 to Eg18), as shown in Table 2. C = control. Polymorphim (arrows) and monomorphic bands (arrows head) are exemplified.

shared by three males (columns 7, 8, 9) showing the same morphological characters (Fig. 1a-c, Table 2).

Recently, Oliveira *et al.* (2004) using RAPD markers to distinguish between two subspecies of *Tetragonisca angustula* Latreille [*Tetragonisca angustula angustula* (Latreille, 1811) and *Tetragonisca angustula fiebrigi* (Schwarz, 1938)] found specific bands to each subspecies of these stingless bees. Also, Waldschmidt *et al.* (2000) identified a RAPD marker that was present in *Melipona quadrifasciata quadrifasciata* Lepeletier and absent in *Melipona quadrifasciata anthidioides* Lepeletier, while Suazo *et al.* (1998) have detected specific RAPD markers which were able to distinguish between *Apis mellifera* L., 1758 races from Africa and Europe. The present findings revealed no specific RAPD marker to any morphological character analyzed. Actually, the absence of correspondence of RAPD data and morphological traits has been reported in the literature (Semagn 2002).

All 127 bands found were used to estimate the similarities among the 18 individuals of *E. truncata*. The mean coefficient of similarity obtained for pairwise comparisons was 0.86 (SD = 0.029), with values ranging from 0.79 to 0.95. The highest similarity (0.95) observed was between the bees Eg5 and Eg16 (both from AF site), while the lowest

(0.79) similarity was between Eg14 (AT) and each of Eg7 (AT) and Eg11 (AF). The mean values of genetic similarities among bees from AT and among bees from AF were, respectively 0.85 (SD = 0.030) and 0.88 (SD = 0.026).

A dendrogram was constructed from the Jaccard coefficients in pairwise comparisons among all the *E. truncata* males, using data pooled from the 12 primers employed (Fig. 2). The values of genetic similarity among clusters of individuals ranged from 0.82 to 0.95, revealing a relatively high genetic similarity among the males of *E. truncata* studied. The highest value found (0.95) was between individuals denoted Eg5 and Eg16 from AF (see the right column - Fig. 2).

The dendrogram did not show distinct clusters of males sharing similar morphological characteristics (Fig. 2). There was a fragile tendency for pairs of bees from the same study area to cluster, such as individuals Eg5 and Eg16, Eg9 and Eg12, Eg3 and Eg15 etc (Fig. 2). However, in general the bootstrap values of branches were low (< 50%), revealing a weakness of the clusters formed. Besides, the branch lengths separating the small and large clusters of individuals were short and showed values of similarity above 80%, indicating thus a high similarity among all individuals. Bees from the same study site did not all group together, suggesting some dissimilarity among them, probably as a result of the genetic variation among individuals within each 'population' (Fig. 2). Furthermore, considering that both study sites were only 3.5 km apart, the levels of genetic similarity found among bees (> 80%) could be indicating that they are moving between both areas. It is known that euglossine bees are able to fly long distances (Janzen 1971), including movements over 4 km across some open habitats (Raw 1989).

Although hair-color and color marks on the integument of bees are not very consistent characteristics for taxonomic studies, they are frequently used in the description of male euglossine species (Dressler 1978, 1982b; Rebêlo & Moure 1995). In the dichotomous key proposed by Rebêlo & Moure (1995), the colour of hairs on the metaepisternum and the marks on the antennal scape of males are included among the main 16 diagnostic characters used to identify of the species described in this study.

Thus, the high genetic similarity revealed by RAPD analysis among the eighteen euglossine males studied indicates that the variations observed in the morphological characters are not in disagreement with the identification of this species of Euglossina and these characters can vary among males of *E. truncata*.

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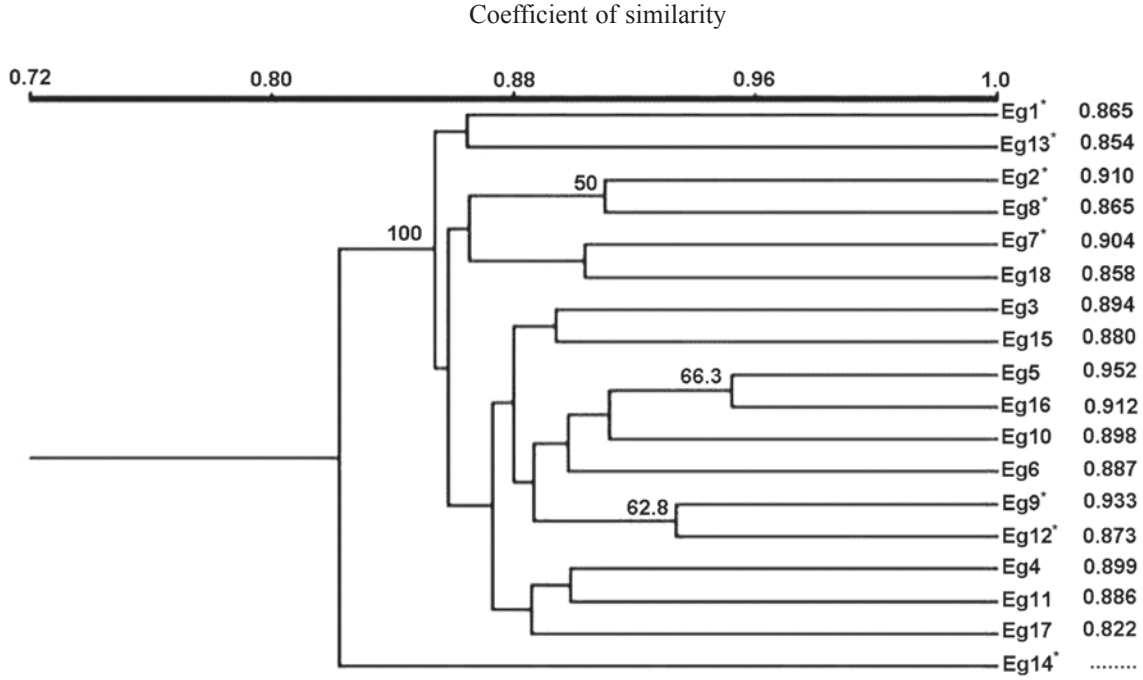

Figure 2. Dendrogram of genetic similarity constructed from the pairwise Jaccard coefficient by the UPGMA method for 18 males of *E. truncata* (denoted Eg1 to Eg18), collected at Parque Arthur Thomas (AT) and in a wooded area around Córrego Água Fresca (AF). * = bees from AT. Numbers at the nodes represent bootstrap generated by 1,000 replications. At dendrogram are shown only the bootstrap values ≥ 50. The similarity measures of consecutive pairs of individuals listed on the dendrogram are shown in the column on the right.

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