SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Evaluation of the RAPD Profiles from Different Body Parts of *Euglossa pleosticta* Dressler Male Bees (Hymenoptera: Apidae, Euglossina)

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Neotropical Entomology 35(6):811-817 (2006)

Avaliação dos Perfis de RAPD de Diferentes Partes do Corpo de Machos de Abelhas *Euglossa pleosticta* Dressler (Hymenoptera: Apidae, Euglossina)

RESUMO - Na literatura atual, são escassas as informações sobre qual a parte do corpo do inseto adulto é mais adequada para a extração de DNA genômico para estudos de análises genéticas baseadas em marcadores de DNA. Neste estudo, foram analisados os perfis de RAPD produzidos a partir da amplificação do DNA genômico extraído de partes distintas do corpo (cabeça, pernas, tórax + asas e abdome) de 12 machos de *Euglossa pleosticta* Dressler. Do total de bandas analisadas, 9,0% não mostraram repetibilidade. As porcentagens de variação de bandas em relação às diferentes partes do corpo das abelhas foram: 1,1% (cabeça); 0,4% (pernas); 0,8% (tórax/asas) e 6,7% (abdome). A maior variação observada ($\chi^2_{\text{para uma amostra}} = 10,27$; gl = 1; $P < 0,01$), nos perfis eletroforéticos de RAPD, produzidos nas amplificações do DNA extraído do abdome de machos euglossíneos sugere que essa parte do corpo de insetos adultos deve ser evitada em procedimentos de extração de DNA. De modo diverso, a baixa variação entre os perfis de RAPD obtidos a partir das amplificações de DNA genômico extraído da cabeça, pernas e tórax/asas das abelhas indica que todas essas partes do corpo de machos euglossíneos são igualmente úteis e confiáveis para serem utilizadas para a extração e a amplificação do DNA genômico.

PALAVRAS-CHAVE: Apoidea, Euglossini, abelha de orquídea, marcador molecular

ABSTRACT - In the current literature, information is scarce on which part of the adult insect body is suitable for isolation of genomic DNA for genetic analysis based on DNA-markers. In this study, we evaluated RAPD profiles generated from total genomic DNA isolated from distinct body parts (head, legs, thorax + wings and abdomen) of 12 males of *Euglossa pleosticta* Dressler. From the total of bands analyzed, 9.0% did not show reproducibility. Percent variations of bands in each body segment were: 1.1% (head); 0.4% (legs); 0.8% (thorax/wings) and 6.7% (abdomen). The much higher variation ($\chi^2_{\text{one sample}} = 10.27$; df = 1; $P < 0.01$) in the RAPD profiles obtained by using DNA isolated from abdomen of the euglossine males suggests that this body part of adult insects should be avoided in DNA extraction procedures. Conversely, the low variation among the RAPD profiles obtained from amplifications of genomic DNA extracted from head, legs and thorax/wings indicates that all these body parts of male bees are equally useful and secure for using in isolation and amplification procedures of total genomic DNA.

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

Recent advances in molecular technology have greatly increased the number of techniques used in genetic studies based on the total genomic DNA analysis. Many of these techniques have been widely employed in genetic analysis of a variety of adult insects (Carvalho & Vieira 2001, Watts *et al.* 2004, Keyghobadi *et al.* 2005, Sofia *et al.* 2005). However, when total genomic DNA is extracted from adult insect body parts, a worrying aspect of the genetic analysis can be the incidence of spurious DNA due, for instance, to the presence of commensal organisms associated with the material under study (Rabouam *et al.* 1999). In researches involving adult bees, or even adult insects in general, the most frequent sources of such DNA are pollen and microorganisms on the insect integument.

Another emerging question when genetic analysis involves adult insects is which part of the insect body is suitable for isolation of the genomic DNA for obtaining reliable DNA-markers. Actually, until this moment there is a gap in the literature concerning this question and, consequently, a wide variation in methods for DNA extraction of adult insects can

be found, including DNA isolation from head (Harper *et al.* 2003), legs (Watts *et al.* 2004), wings or the whole insect (Keyghobadi *et al.* 2005).

Among the most known techniques based on the analysis of total genomic DNA, RAPD (Random Amplified Polymorphic DNA) has been described as a simple and easy method of detecting polymorphism based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al.* 1990). Because RAPD is a fast and technically simple method, requiring minimal amounts of DNA and no previous knowledge of the genome (Williams *et al.* 1990, Ayliffe *et al.* 1994), it has been used in many different biological approaches, such as mapping (Martin *et al.* 1991), identification of species or subspecies (Hillis *et al.* 1996), parentage analysis (Hunt & Page 1992) and evaluation of anthropogenic stress in aquatic ecosystems (Bickham *et al.* 2000). In addition, RAPD markers are relatively inexpensive to produce and can be visualized and scored on regular agarose gels (KjØlner *et al.* 2004).

On the other hand, according to several authors, the reproducibility between RAPD reactions can be hard to maintain (Ayliffe *et al.* 1994, Rabouam *et al.* 1999) since small changes in reaction conditions, such as the type of DNA polymerase used, template DNA concentration, magnesium concentration and temperature-profiling characteristic of the thermalcycler can produce changes in RAPD bands profiles (Pérez *et al.* 1998). Thus, possible solutions proposed to minimize problems in RAPD reproducibility include a careful optimization of the concentrations of components in the reaction mixture and of the amplification conditions of the thermal cycler, which should be consistently maintained throughout the study (Chiappero & Gardenal 2001).

In this context, our central aim was to investigate the occurrence of variations in RAPD profiles produced by amplification of total genomic DNA isolated from different body parts of adult male bees.

Material and Methods

Sample collections. Samplings were carried out during March of 2004, at Mata dos Godoy State Park, a remnant of Atlantic Rainforest located in northern Paraná State, southern Brazil. After being attracted to scent baits of eucalyptol, 12 males of *Euglossa pleosticta* Dressler were collected with an insect net, placed individually in plastic tubes, transported alive to the laboratory and stored frozen (-20ºC) until DNA extraction. All bees were identified by S. H. Sofia and voucher specimens are deposited at the Museu de Zoologia, Universidade Estadual de Londrina (MZUEL).

DNA extraction and RAPD analysis. The following insect body parts were separated for subsequent RAPD analysis: a) head, b) six legs, c) thorax plus wings and d) abdomen. Prior to DNA isolation, bees were submitted to two distinct pre-treatments, to remove exogenous material (e.g. fungal spores or grains of pollen) possibly attached to the bee integument, as follows: each body part of bees was rinsed individually in a sterilized plate with distilled water or 70% alcohol. The methodology of total genomic DNA extraction used was based on Sofia *et al*. (2005). DNA concentration was determined in a fluorometer (200 DyNA Quant, Hoefer), using the dye Hoechst 33258, and then all DNA samples were diluted to a standard concentration (5ng/µl). All isolates were stored at -20°C.

The RAPD profiles were generated from total genomic DNA according to Sofia *et al.* (2005). Amplification reactions were carried out in a final volume of 15 µl containing 15-25 ng of template DNA, 250 µM dNTPs (Pharmacia), 0.3 µM of ten-nucleotide primer (Operon Technologies, Alameda, CA, USA), 4.0 mM $MgCl_2$ and 0.6 µl (3 units) of DNA polymerase in the reaction buffer supplied. The following primers were used in the analysis: OPX-04, OPX-06, OPX-16 and OPAM-14. Control reactions were run containing all components except genomic DNA. DNA amplifications were carried out in a PTC-100, MJ Research thermal cycler and the amplification protocol consisted of 4 min denaturation at 92°C followed by 40 cycles of 40 at 92°C, 1.5 min at 40°C, and 2 min at 72°C, and a final cycle of 5 min at 72°C.

Samples of 15 µl of the amplification products were separated by electrophoresis on 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), run at $3V.cm^{-1}$, visualized with ethidium bromide staining and photographed under UV light with the Kodak Digital Science system (EDAS120).

Data analysis. To test differences between the two pre-treatments, two groups of treatments were made. In the first, head and legs from the same bee were washed, individually, in distilled water, while thorax/wings and abdomen were washed, separately, with 70% alcohol. In the second group, these treatments were inverted, i.e., head and legs from the same bee were washed individually with alcohol 70%, while thorax/wings and abdomen were rinsed, independently, with distilled water.

Comparative analyses were carried out by placing all samples on the same gel. Additionally, the samples (head, legs, thorax/wings and abdomen) from the same individual were placed side by side on the gel. RAPD marker profiles were determined by direct comparison of the amplified DNA electrophoretic profiles, and each sample was scored for the presence or absence of amplification products (binary variable).

The chi-squared test (2 x 2) was applied to compare the two pre-treatments (washing in alcohol or water). An one-sample χ^2 test was employed to compare possible differences in RAPD profiles from different groups of body parts, as follows: 1) head; 2) legs; 3) thorax +wings and, 4) abdomen submitted to each pre-treatment. Values were considered different when $P < 0.05$ (Siegel 1981).

The estimate of reproducibility among body samples isolated from the same bee (R_{xywz}) was calculated by dividing the number of bands common to all four segments (x, y, w, z) in the RAPD profile (n_{xywz}) by the average number of scored bands for all body parts, viz.: $R_{xywz} = 4n_{xywz} / (n_x + n_y + n_w + n_z)$, where n_x, n_y, n_w and n_z represent the number of RAPD bands in head, legs, wings+thorax and abdomen, respectively (Pérez *et al*. 1998). Samples that partially or totally failed in the amplification were not included in the analysis.

After calculation of the reproducibility index for each bee with each primer, a mean value of the reproducibility including the 12 individuals analyzed was estimated.

A pair-wise similarity matrix was constructed using the Jaccard (*J*) index (Sneath & Sokal 1973). On the basis of the *J*–values of the samples, the UPGMA clustering method was followed, employing the NTSYS-PC package (Rohlf 2000) to generate a dendrogram of similarity. To evaluate the robustness of the groupings formed, a bootstrap analysis, with 1000 replications, was performed using the Bood software program (Coelho 2000).

DNA fragment sizes were estimated by comparison to the standard Ladder 100 bp (Biotools), and by using the GEL software.

Results

The RAPD marker patterns were assessed in *E. pleosticta* DNA extracted from different body parts of 12 males, totalling 48 samples. The largest number of bands was found with primer OPX-06. The four primers used produced from 9 to 25 bands, which varied from 371 to 4053 bp (Table 1). Reproducibility of the results was tested with primer OPX-16 and no variation was found. The average reproducibility index estimated for each primer varied from 0.92 to 0.98 (Table 1), revealing a high similarity among the profiles for each body part from the same bee analyzed.

Fig. 1 shows the RAPD profiles for 48 samples amplified with primers OPX-06, OPX-04 and OP-AM14. In these profiles, it is possible to observe the high reproducibility among the four different body parts from each bee. Also, a negative control from RAPD reactions amplified with the primer OPAM-14 produced some faint bands, which were mostly different from bands in the amplified reactions (Fig. 1).

The comparative analysis of RAPD profiles revealed no significant difference ($\chi^2 = 2.44$, df = 1; $P > 0.05$) between the two pre-treatments (water or alcohol). Therefore, to reveal any differences between the RAPD profiles from the four different groups of body parts (head, legs, thorax + wings, abdomen), both pre-treatments were grouped and the profiles produced by each body part were compared for each bee individually.

Out of a total of 476 fragments analyzed, 9.0% (n = 43) did not show reproducibility (Table 2). Percentages of variation in the reproducibility for the different body segments were: 1.05% (head); 0.42% (legs); 0.84% (thorax/wings) and 6.72% (abdomen). Application of the χ^2 test (one sample) revealed significant differences among these occurrences of variations ($\chi^2 = 56.44$, df = 3; $P < 0.001$), indicating some variation in amplified products from the same individual.

The dendrogram of similarity obtained by applying the Jaccard index and the UPGMA method revealed that different parts from the same individual clustered together, showing coefficients ranging from 0.84 to 1.0 (Fig. 2). Bootstrap values for the UPGMA tree were generally very high (> 95%) for the branches clustering different body parts of the same bee, reflecting a high robustness of these branches. The lowest value observed (0.84) occurred between the abdomen from individual 1 and the other body segments of this bee. However, for the majority of individuals analyzed, the values of similarity among the different body parts from the same bee were above 0.9, indicating the high reproducibility of RAPD technique. After clustering together, the four body parts from the same bee formed new clusters with other individuals. The similarity among individuals ranged from 0.60 to 0.81.

Discussion

Among the 43 occurrences that did not show complete reproducibility four (9.3%) were due to the presence of bands that were absent in other samples (Table 2), and 39 occurrences (90.7%) corresponded to the absence of bands in one or two samples from the same individual.

Our results showed a high reproducibility of RAPD profiles produced by different body parts from the same bee (Fig. 1 and Table 2), suggesting that when optimization and standardization of RAPD technique are undertaken, reproducible profiles can be satisfactorily achieved. The differences in repeatability shown by body segments ($\chi^2 = 56.44$, df = 3; $P < 0.001$) were possibly due to variations in abdomen profiles, since this body part showed a lower repeatability (6.7% of variation) than the other insect body segments. Besides, when variation in abdomen profiles (n = 32) was compared to the total number of variations shown by other body parts (n = 11), a significant difference was found ($\chi^2_{\text{one sample}} = 10.27$, df = 1; $P < 0.01$). In part, the greater variation in abdomen profiles may be attributed to low reproducibility shown by the abdomen from individuals 1 and 4 (Table 2). In part, in both situations, DNA obtained from abdomen might have suffered damages during the isolation phase, since these samples showed several absences in RAPD products obtained

Table 1. Nucleotide sequences from primers used in RAPD reactions, number of amplified fragments and range of fragment sizes produced by different primers and average reproducibility estimates.

Primer	Nucleotide sequence	Number of fragments	Range of fragment size (bp)	Reproducibility (R_{xywz})
OPX-04	5' CCGCTACCGA 3'	14	524 - 3370	0.95
OPX-06	5' ACGCCAGAGG 3'	25	371 - 4053	0.92
OPX-16	5' CTCTGTTCGG 3'	9	706 - 2366	0.98
OPAM-14	5' TGGTTGCGGA 3'	21	374 - 2806	0.94

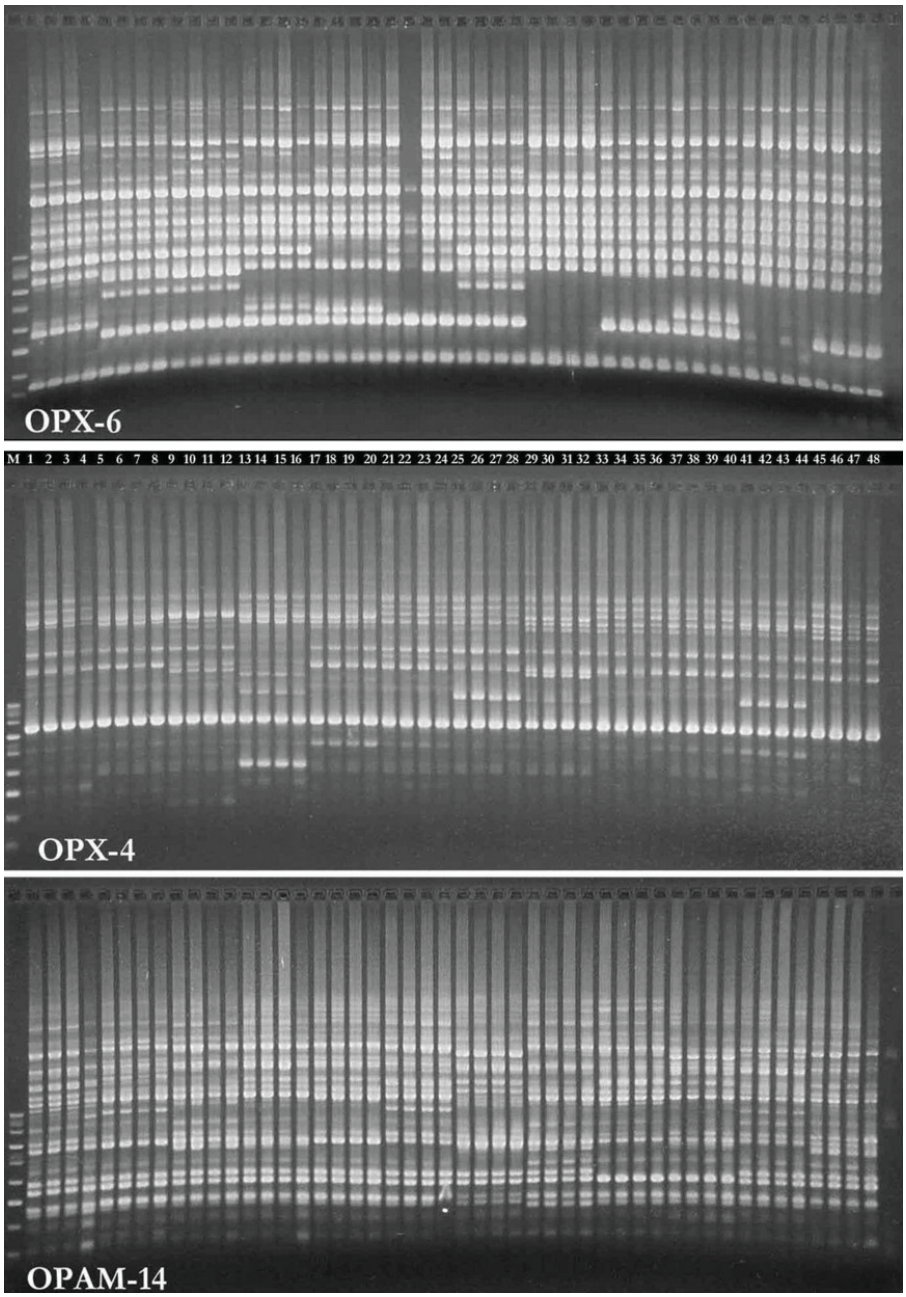

Fig. 1. RAPD profiles of *E. pleosticta*, from different body segments, amplified with primers OP-X6, OP-X4 and OP-AM14. Lane M = 100bp molecular weight marker. Samples of the same bee were arranged in the gel as follows: head, legs, thorax + wings and abdomen. Lanes: 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, 27, 28, 31, 32, 35, 36, 39, 40, 43, 44, 47, 48 = parts of bees washed with water. Lanes: 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, 24, 25, 26, 29, 30, 33, 34, 37, 38, 41, 42, 45, 46 = parts of bees washed with alcohol. Last lane = control.

with all four primers used in amplifications reactions (Table 2 and Fig. 1). Regardless of their feeding habits, all insects possess a range of enzymes in the midgut, located in the abdomen, where digestion occurs (Chapman 1982). According to Walker *et al.* (1998), proteases are present in the gut of adult insects and show activity over a wide pH range. Thus, some of the variations in abdomen RAPD profiles may be attributed to the action of digestive enzymes (eg. esterase) on DNA during the extraction stage.

Rabouam *et al.* (1999) attributed artefactual RAPD polymorphism to the presence of commensal organisms and fragment rearrangements during PCR amplification.

Table 2. Features of the RAPD fragments produced by amplification of genomic DNA isolated from individual body parts of *E. pleosticta* males that did not show reproducibility with other body parts.

Number of bee	Fragment name	Body part	Size (bp) of RAPD fragment	Kind of variation	Treatment
1	B2-X06	Abdomen	3963	Absence	A
1	B7-X06	Abdomen	2566	Absence	A
1	B8-X06	Thorax	2501	Absence	A
1	B8-X06	Abdomen	2501	Absence	A
1	B11-X06	Abdomen	2023	Absence	A
1	B1-AM14	Abdomen	2806	Absence	A
1	B7-AM14	Abdomen	1696	Absence	A
1	B12-AM14	Abdomen	1075	Presence	A
2	B2-AM14	Abdomen	2427	Absence	A
2	B14-AM14	Leg	846	Absence	W
3	B1-X04	Abdomen	3370	Absence	A
3	B1-X06	Abdomen	4053	Absence	A
3	B2-X06	Abdomen	3963	Absence	A
3	B3-X06	Abdomen	3648	Absence	A
3	B11-X06	Abdomen	2023	Absence	A
3	B13-X06	Abdomen	1471	Absence	A
4	B2-X06	Abdomen	3963	Absence	A
4	B4-X06	Abdomen	3501	Absence	A
4	B5-X06	Abdomen	3282	Absence	A
4	B8-X06	Head	2501	Absence	W
4	B8-X06	Abdomen	2501	Absence	A
4	B16-X06	Head	1103	Absence	W
4	B1-AM14	Abdomen	2806	Absence	A
4	B14-AM14	Abdomen	846	Absence	A
4	B17-AM14	Abdomen	605	Absence	A
5	B2-X16	Head	2207	Absence	W
5	B2-X16	Abdomen	2207	Absence	A
5	B17-AM14	Thorax	605	Presence	A
5	B17-AM14	Abdomen	605	Presence	A
6	B5-X06	Head	3282	Absence	W
7	B9-X06	Thorax	2477	Absence	W
7	B20-X06	Leg	862	Absence	A
7	B20-X06	Abdomen	862	Absence	W
9	B7-X04	Thorax	1868	Absence	W
9	B13-AM14	Abdomen	977	Absence	W
9	B17-AM14	Abdomen	605	Absence	W
11	B6-X04	Abdomen	2438	Absence	W
11	B5-X06	Head	3282	Absence	A
11	B9-X06	Abdomen	2477	Presence	W
12	B6-AM14	Abdomen	1821	Absence	W
12	B7-AM14	Abdomen	1696	Absence	W
12	B15-AM14	Abdomen	769	Absence	W
12	B15-AM14	Abdomen	769	Absence	W

W = water; A = alcohol; the number of bee corresponds to its position on the gel; fragment name is represented by number of band (ex. B15 = 15th band), showed in descending size, followed by the corresponding primer.

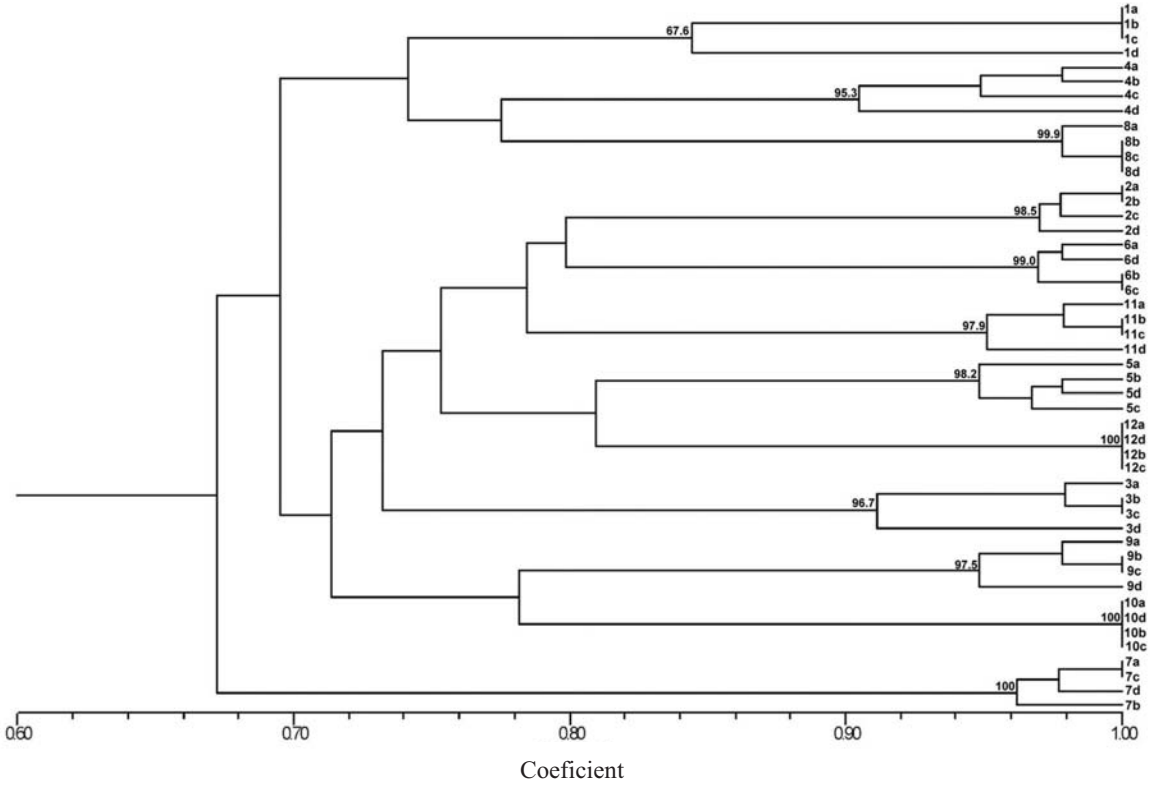

Fig. 2. Dendrogram of genetic similarity constructed using the Jaccard coefficient and the UPGMA method for different body parts of 12 males of *E. pleosticta*. Numbers from 1 to 12 represent each bee. a = head, b = legs, c = thorax + wings and d = abdomen. Numbers at the nodes represent bootstrap values generated by 1000 replications.

According to these authors, artefacts accounted for 50-75% of all the RAPD fragments obtained, and they concluded that it is impossible to estimate the degree of DNA polymorphism by RAPD studies. Our results suggest that the absence of reproducibility did not result from exogenous material, such as pollen or microorganisms, in the gut of bees, since only in four cases additional bands were observed in RAPD profiles from the same individual (Table 2). In contrast to males of eusocial *Apis mellifera* L. (Hymenoptera: Apidae), which stay inside the nest for several days after emergence (Winston 1987), most reports indicate that euglossine males leave the nest promptly and do not return (Dodson 1966). During adult life, euglossine males need only nectar supplies (Dodson 1966), which provide energy for their activities. For this reason, the presence of pollen in the digestive tube of euglossine males is very improbable.

The four additional bands produced in RAPD reactions with primers OPX-06 and OPAM-14 (Table 2) probably are artefacts, since such bands may arise from heteroduplex formation between amplified products (Ayliffe *et al.* 1994) or from other secondary artifacts (Hadrys *et al.* 1992). Also, the few faint bands observed in the negative control, amplified with primer OPAM-14 (Fig. 1) were attributed to heteroduplex molecules (Ayliffe *et al.* 1994). The presence of such bands in the negative control were already reported by other authors (Williams *et al.* 1990, Pérez *et al.* 1998).

Overall, the current results indicate that the much higher variation observed in abdomen profiles ($\chi^2_{\text{one sample}}$ = 10.27; df = 1; $P < 0.01$) suggests that this body segment of bees and probably other insects should be avoided in DNA extraction procedures. Conversely, the low variation among RAPD profiles obtained from amplifications of head, legs and thorax/wings indicates that all these body parts of male bees are equally useful and secure for using in isolation and amplification procedures of total genomic DNA.

Acknowledgments

The authors are grateful to IAP (Instituto Ambiental do Paraná) and IBAMA (Instituto Brasileiro do Meio Ambiente) for permission to collect male euglossine bees at Parque Estadual Mata dos Godoy and to the anonymous reviwers for comments on the manuscript. ANT Pascual and KM Suzuki thank CAPES for providing the scholarship. This study was supported by grants provided by FBPN (Fundação O Boticário de Proteção à Natureza) and Fundação Araucária and ProDiCi/UEL.

References

Ayliffe, M.A,. G.J. Lawrence, J.G. Ellis & A.J. Prior. 1994. Heteroduplex molecules formed between allelic sequences cause

nonparental RAPD bands. Nucleic Acids Res. 22: 1632-1636.

Bickham, J.W., S. Sandhu, P.D.N. Hebert, L. Chickhi & R. Athwal. 2000. Effects of chemical contaminants on genetic diversity in natural populations: Implications for biomonitoring and ecotoxicology. Mutat. Res. 463: 33-51.

Carvalho, A.O.R. & L.G.E. Vieira. 2001. Determinação das condições ótimas para análises de PCR-RAPD em *Atta sexdens rubropilosa* Forel (Hymenopera: Formicidae). Neotrop. Entomol. 30: 593-600.

Chapman, R.F. 1982. The insects: Structure and function, 3rd ed. London, Hodder and Stoughton Ltd., 934p.

Chiappero, M.B. & C.N. Gardenal. 2001. Inheritance of random amplified polymorphic DNA (RAPD-PCR) markers and their use in population studies of *Calomys musculinus* (Rodentia, Muridae), the reservoir of Argentine Hemorrhagic Fever. Hereditas (Lund) 135: 85-93.

Coelho, A.S.G. 2000. BOOD: Avaliação de dendrogramas baseados em estimativas de distâncias/similaridades genéticas através do procedimento de bootstrap. Software, Goiânia, UFG.

Dodson, C.H. 1966. Ethology of some bees of the tribe Euglossini (Hymenoptera: Apidae). J. Kans. Entomol. Soc. 39: 607-629.

Hadrys, H., M. Balick & B. Schierwater. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1: 55-63.

Harper, G.L., N. McClean & D. Goulson. 2003. Microsatellite markers to assess the influence of population size, isolation and demographic change in the genetic structure of the UK butterfly *Polyommatus bellargus*. Mol. Ecol. 12: 3349-3357.

Hillis, D.M., C. Moritz & B.K. Mable (eds). 1996. Molecular systematics, 2nd ed. Sunderland, Cumbria, Sinauer Associates, Inc., 655p.

Hunt, G.J. & and R.E. Page Jr. 1992. Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee. Theor. Appl. Genet. 85: 15-20.

Keyghobadi, N., J. Roland & C. Strobeck. 2005. Genetic differentiation and gene flow among populations of the alpine butterfly, *Parnassius smintheus*, vary with landscape connectivity. Mol. Ecol. 14: 1897-1909.

KjØlner, S., S.M. Såstad, P. Taberlet & C. Brochmann. 2004. Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: Clonal diversity in *Saxifraga cernua*. Mol. Ecol. 13: 81-86.

Martin, G.B., J.G.K. Williams & and S.D. Tanskley. 1991. Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc. Natl. Acad. Sci. U.S.A. 88: 2336-2340.

Pérez, T., J.A. Albornoz & A. Domênguez. 1998. An evaluation of RAPD fragment reproducibility and nature. Mol. Ecol. 7: 1347-1357.

Rabouam, C., A.M. Comes, V. Bretagnolle, J-F. Humbert, G. Periquet & Y. Bigot. 1999. Features of DNA fragments obtained by random amplified polymorphic DNA (RAPD) assays. Mol. Ecol. 8: 493-503.

Rohlf, F.J. 2000. NTSYSpc, numeral taxonomy and multivariate analysis system, version 2.1. Exeter Software, Applier Biostatistics, Setauket, New York.

Siegel, S. 1981. Estatística não-paramétrica: Para as ciências do comportamento. São Paulo, Ed. McGraw-Hill do Brasil, Ltda., 350p.

Sneath, P.H.A. & R.R. Sokal. 1973. Numerical taxonomy: The principles and practice of numerical classification. San Francisco, California, WH Freeman, 573p.

Sofia, S.H. F.M. Paula, A.M. Santos, F.S. Almeida & L.M.K. Sodré. 2005. Genetic structure analysis of *Eufriesea violacea* (Hymenoptera, Apidae) populations from southern Brazilian Atlantic Forest remnants Genet. Mol. Biol. 28: 479-484.

Walker, A.J., L. Ford, M.E.N. Majerus, I.E. Geoghegan, N. Birch, J.A. Gatehouse & A.M.R. Gatehouse. 1998. Characterisation of the mid-gut digestive proteinase activity of the two-spot ladybird (*Adalia bipuntacta* L.) and its sensitivity to proteinase inhibitors. Insect Biochem. Mol. Biol. 28: 173-180.

Watts, P.C., R. Rouquette, I.J. Saccheri, S.J. Kemp & D.J. Thompson. 2004. Molecular and ecological evidence for small-scale isolation by distance in an endangered damselfly, *Coenagrium mercuriale*. Mol. Ecol. 13: 2931-2945.

Williams, J.G.K., A.R. Kubelik, K.L. Livak & S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Winston, M.L. 1987. The biology of the honeybee. Cambridge, Massachussets, Harvard University Press, 281p.

Received 03/III/06. Accepted 04/VII/06.