

GENETIC VARIABILITY IN NATURAL POPULATIONS OF BRAZILIAN SOCIAL BEES: II. ELECTROPHORETIC DATA FOR PGM AND MDH GIVE EVIDENCE FOR MULTIPLE FERTILIZATIONS IN STINGLESS BEES

T.M.M.A Falcão¹ and E.P.B. Contel²

ABSTRACT

Of nine species studied for PGM, two showed genetic variation: *Plebeia droryana* and *Scaptotrigona bipunctata*. The polymorphism detected in *P. droryana* is controlled genetically by three alleles, whereas the polymorphism of *S. bipunctata* is controlled by only two alleles at one locus. Experimental evidence indicates that in these organisms the structure of PGM is monomeric. Of ten species studied for MDH, three were polymorphic: *Melipona marginata marginata*, *Partamona (Partamona) helleri* and *P. droryana*. Two alleles at one locus genetically control the MDH polymorphism in *M. m. marginata* and *P. (P.) helleri*, whereas the MDH variability observed in *P. droryana* is regulated by three alleles at one locus. Analysis of genotypic proportions for MDH in the progenies of segregant crosses of *P. droryana* revealed statistical significance. Two hypotheses are raised to explain this discrepancy: the coexistence of more than one egg-laying queen in a single nest and/or the occurrence of multiple fertilizations of the egg-laying queen.

INTRODUCTION

The meliponids, Brazilian stingless bees, belong to the class Insecta, order Hymenoptera, superfamily Apoidea, family Apidae, subfamily Meliponinae, and tribe Meliponini.

¹ Departamento de Biologia, Área de Genética, Universidade Federal Rural de Pernambuco, 50000 Recife, PE, Brasil.

² Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, 14049 Ribeirão Preto, SP, Brasil.
Send correspondence to E.P.B.C.

All the three known types of parthenogenesis, i.e. apomictic, automictic and generative or haploid, are represented in the order Hymenoptera (Suomalainen, 1962). The type described in bees is the generative one, whereby the egg can develop with or without fertilization. Fertilized eggs give origin to diploid females (queens and workers), and the unfertilized ones produce males (drones).

The haplodiploidy which characterizes these insects turns them into very valuable material for genetic studies, since the allelic segregation observed in males for any locus directly reflects the constitution of the female which generated them.

A great diversity of life patterns, niches occupied and evolution of social forms exists among hymenopterans (Crozier, 1977; Wille, 1983). Among the biological aspects of meliponids summarized by Contel (1972) and Del Lama (1977) which are of interest for the present study, we emphasize the evidence that a *Melipona* queen mates with a single male and that in each meliponid hive, only a single fecundated queen, a few virgin queens, many workers and some drones may coexist. However, the protein polymorphism data presented in the present investigation indicate the possibility that a meliponid queen may undergo multiple fertilizations.

MATERIAL AND METHODS

Ninety-seven and 64 colonies were sampled for NAD-dependent malate dehydrogenase (MDH) and phosphoglucomutase (PGM), respectively. The number of females and males analyzed for the two enzymes was 1036 and 168, and 704 and 87, respectively.

The data were obtained from analyses of homogenates of black-eyed and white-bodied pupae of 10 species of meliponids, from various areas of four Brazilian states, i.e.:

Melipona compressipes - São Luiz, State of Maranhão

M. marginata marginata - Prudentópolis, State of Paraná, and Pocinhos do Rio Verde, State of Minas Gerais

M. quadrifasciata anthidioides - Pocinhos do Rio Verde, State of Minas Gerais

Plebeia droryana - Ribeirão Preto, State of São Paulo, Londrina and Prudentópolis, State of Paraná

Friesella schrottkyi - Londrina and Prudentópolis, Paraná

Partamona (Partamona) helleri - Ribeirão Preto, São Paulo

Scaptotrigona postica postica - Ribeirão Preto, São Paulo

S. bipunctata - Londrina, Paraná and Pocinhos do Rio Verde, Minas Gerais

Tetragonisca angustula - Londrina, Prudentópolis and Sete Quedas, State of Paraná

Nannotrigona testaceicornis testaceicornis - Londrina, State of Paraná

The *M. compressipes* were not analysed for PGM.

The samples were homogenized individually in small test tubes in a volume of twice-distilled water proportional to the size of the bee, and centrifuged at 3,000 rpm for 10 minutes at room temperature. The supernatants were absorbed with a Whatman no. 3 filter paper (5 x 6 mm pieces for MDH analysis and 1 x 7 mm pieces for PGM) and applied to the gels.

NAD-Dependent malate dehydrogenase (EC 1.1.1.37)

MDH was studied on an 11% starch gel, horizontal system, prepared with 0.02 M Tris-HCl buffer, pH 8.6. The buffer used in the cuvettes was 0.3 M Tris-HCl, pH 8.6. In the first analyses we added NAD to the gel (20 mg/100 ml gel), and electrophoresis lasted 16 hours at a temperature of approximately 8°C, with the gel submitted to a voltage gradient of 2.7 V/cm. In later runs, NAD was no longer added, the time for electrophoresis was reduced to 6 hours and the gel was submitted to a voltage gradient of 6.7 V/cm, with good resolution obtained. The MDH activity bands were visualized using sodium L-malate as a substrate according to the method described by Contel *et al.*, 1977.

Phosphoglucomutase (EC 5.4.2.2)

For the PGM analyses we used agarose-starch gel (0.8% and 2%, respectively) and a horizontal system. The buffer used in the cuvettes was Tris-EDTA-maleic acid-magnesium chloride, pH 7.4 (0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA and 0.01 M magnesium chloride). The same buffer at 1:15 dilution was used to prepare the gel. Electrophoresis was performed for 5 hours, at a temperature of approximately 8°C and with the gel submitted to a voltage gradient of 4.8 V/cm.

The reaction mixture was prepared with 35 mg of the substrate α -D-glucose-1-phosphate (containing approximately 1% glucose-1,6-diphosphate), 10 ml Tris-magnesium chloride buffer, pH 8.0 (0.1 M Tris and 0.02 M MgCl₂), 0.4 ml NADP solution, 5 μ l glucose-6-phosphate dehydrogenase (300 units/mg protein), 0.5 ml MTT, 0.5 ml PMS, and approximately 10 ml 2% agar previously dissolved in distilled water. The NAD, NADP, MTT and PMS solutions were prepared at the proportion of 5 mg/ml water.

RESULTS

The nomenclature used in the present paper is that proposed by Shows *et al.* (1979) for human genes.

MDH genetic polymorphisms were detected in 3 of the 10 species studied, i.e. *M. m. marginata*, *P. droryana* and *P. (P.) helleri*. The remaining species showed no genetic variation.

Figure 1A shows the two MDH electrophoretic phenotypes detected in samples of *M. m. marginata*. The MDH1 1 phenotype was not detected in our samples.

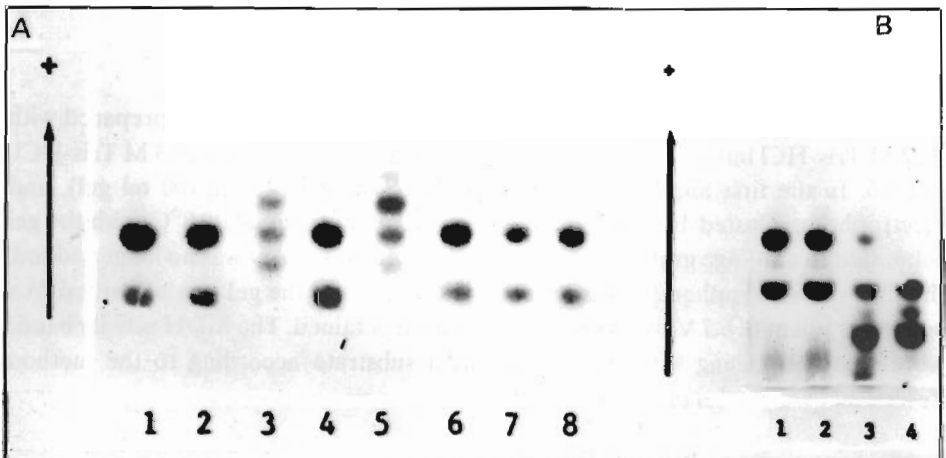


Figure 1 - Variation in NAD-dependent malate dehydrogenase at pH 8.6 observed in *M. marginata marginata* (A) and *P. (Partamona) helleri* (B). A. Samples 3 and 5 are from worker pupae having the MDH1 1-2 phenotype, and the remaining ones are MDH1 2. B. Worker pupae having the MDH1 1 phenotype (1 and 2) and the MDH1 1-2 (3) phenotype. Sample 4 is of a *P. droryana* worker pupae with the MDH1 3 phenotype.

The variation in MDH observed in *P. (P.) helleri* is illustrated in Figure 1B. The variation in the MDH electrophoretic phenotypes of *M. m. marginata* and *P. (P.) helleri* indicates that at least two alleles at one locus are responsible for this polymorphism in each case.

Figure 2 shows the phenotypes detected in *P. droryana* samples. Five phenotypes were detected among females and only three phenotypes were detected among males: MDH1 1, MDH1 2 and MDH1 3. These results suggest that the genetic control of MDH polymorphism in *P. droryana* is exerted by three alleles: *MDH1*1*,

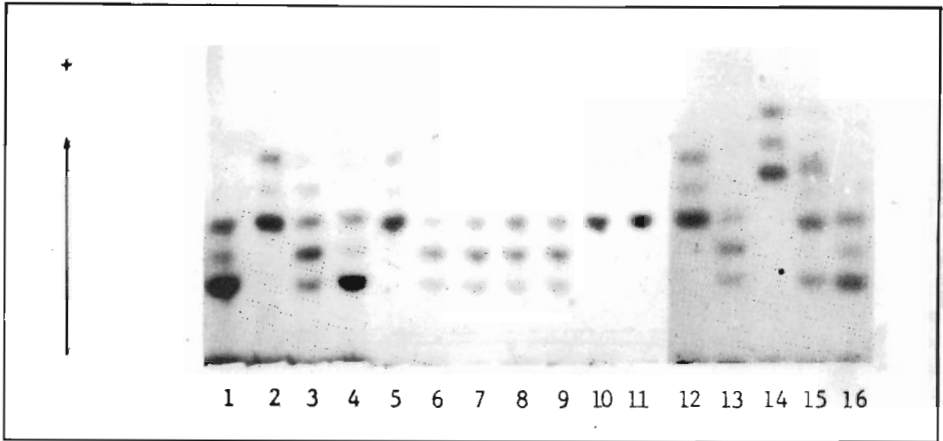


Figure 2 - MDH polymorphism at pH 8.6 observed in *P. droryana*. Samples 1 to 11 are worker pupae from a single hive: MDH1 2 (2,5,10 and 11), MDH1 3 (1 and 4) and MDH1 2-3 (3, 6 to 9). Samples 12 to 16 show phenotypes MDH1 2 (12), MDH1 1 (14), MDH1 1-3 (15) and MDH1 3 (16). Sample 13 is from an MDH1 2-3 adult.

*MDH1*2* and *MDH1*3*. Since the males of these organisms are haploid, only three phenotypes are expressed in them, each reflecting the action of one allele.

Samples 1 to 11 shown in Figure 2 are from females of a single colony. Segregation of two types of homozygotes in addition to the heterozygote indicates that the queen of this colony may have been fecundated by more than one drone or, alternatively, that two egg-laying queens can live harmoniously together in a single nest.

Polymorphism for phosphoglucumutase was detected only in two of the nine species analyzed for this system: *P. droryana* and *S. bipunctata*. Three phosphoglucumutase activity bands were observed in *P. droryana* and named fast, slow and ultraslow according to their electrophoretic mobility. The three bands are genetically determined by alleles *PGM*1*, *PGM*2* and *PGM*3*, respectively.

Five phenotypes were detected in worker pupae, only four of which are shown in Figure 3. The figure also shows the phenotypes observed in males. Phenotype PGM 3 corresponding to genotypes *PGM*3* of males and *PGM*3/PGM*3* of females was not detected in our samples.

Two phenotypes were detected in worker pupae of *S. bipunctata*, one of them consisting of two electrophoretic fractions and denoted PGM 1-2, and the other consisting only of the more anodal electrophoretic fraction of phenotype PGM 1-2 and denoted PGM 1.

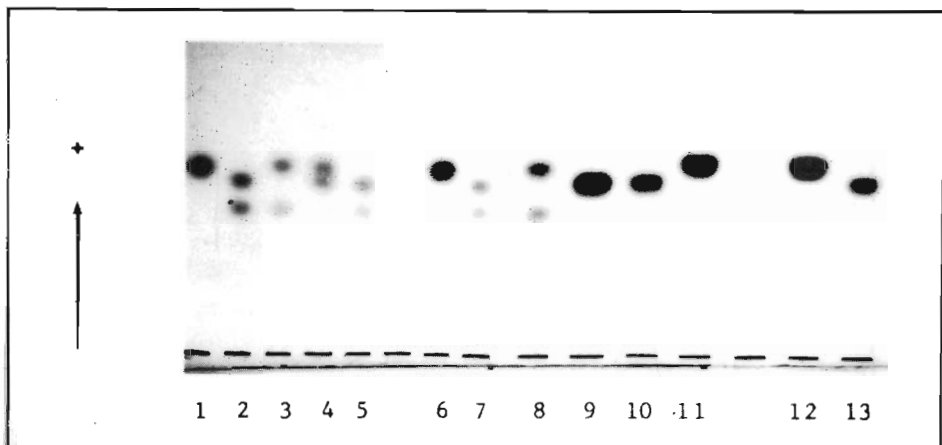


Figure 3 - Variation observed for phosphoglucosmutase at pH 7.4 in *P. droryana*. Samples 1 to 8 are worker pupae: PGM 1 (1 and 6), PGM 1-2 (4), PGM 1-3 (3 and 8) and PGM 2-3 (2, 5 and 7). Samples 9 to 13 are drone pupae: PGM 1 (11 and 12) and PGM 2 (9, 10 and 13).

In Figure 3, samples 1 to 8 are from workers and 9 to 12 from drones of a single hive. The detection of four different phenotypes among females does not fit the information that *Melipona* queens are fertilized only by a single male (Kerr and Krause, 1950; Contel and Kerr, 1976). The distribution of PGM and MDH electrophoretic phenotypes obtained in the present study for individuals from a single hive cannot be explained on the basis of a single mating, because the males of these organisms are haploid.

In *P. droryana* and *F. schrottkyi*, a differential relationship was observed between PGM activity and ontogenetic development of the samples. Larvae, black-eyed pupae, one-day old adults and flying adults were analyzed for the two species. Intensified staining of phosphoglucosmutase activity bands was observed in *P. droryana* larvae, but no activity was detected in adults able to fly. In contrast, the staining intensity of the isozyme components of *F. schrottkyi* was equivalent in larvae, black-eyed pupae and one-day old adults and lower than that observed in adults able to fly.

Statistical tests were performed to determine the agreement between observed and expected genotypic proportions in the progenies of informative crosses for MDH and PGM. The chi-square test was used, with the Yates correction performed whenever there was one degree of freedom and an expected value lower than 5 (Stansfield, 1969; Daniel, 1974; Siegel, 1975). Statistical analysis was applied only to the cases for which we had evidence of the involvement of a single drone in the fertilization of the queen.

As shown in Tables I and II, the nonsignificant chi-square values obtained indicate that the genetic segregations for the loci in question were in agreement with theoretically expected Mendelian proportions, except for the segregation obtained for the *MDH1*1/MDH1*3* x *MDH1*1* cross of *P. droryana*, a sample from Londrina, Paraná ($\chi^2 = 10.286$; $P < 0.01$).

DISCUSSION

The electrophoretic data obtained in the present study suggest that the phosphoglucosyltransferase of the organisms studied has a dimeric structure. In *P. droryana*, PGM polymorphism is controlled genetically by a locus with at least three alleles, and in *S. bipunctata* by a locus with two alleles.

The data obtained in the present study about NAD-dependent malate dehydrogenase are not sufficient to permit inferences about the structure of the molecule of this enzyme. In this case, the complexity of electrophoretic patterns was greater than that observed for phosphoglucosyltransferase. This complexity is well illustrated in Figure 2, where it can be seen that the homozygote patterns for alleles *MDH1*1*, *MDH1*2* and *MDH1*3* exhibit three activity bands, whereas heterozygotes have electrophoretic phenotypes consisting of five bands (*MDH1*2/MDH1*3* individuals) or four (*MDH1*1/MDH1*3* individuals). On the other hand, only three components are visualized in heterozygous adults (Figure 2, sample 13).

Previous studies by Contel *et al.* (1977) led these authors to propose a dimeric structure for MDH of bees. Evidence of a dimeric structure for *Apis mellifera* MDH has been obtained by Snyder *et al.* (1979) and by Del Lama (1982), as well as for MDH of other organisms such as *Tetrahymena pigmentosa* (Preparata, 1983) and maize (Goodman *et al.*, 1980).

The estimate of intralocus heterozygosity for MDH for the 10 meliponid species studied was 0.10, and an identical value was obtained for PGM in 9 of the 10 species. Estimates of intralocus heterozygosity for MDH for 28 *Drosophila* species and for four species of insects of the genera *Philaenus*, *Gryllus* and *Magicicada* are 0.060 and 0.012, respectively; intralocus heterozygosity values for PGM for the same species are 0.212 and 0.279, respectively (data compiled by Selander, 1976).

In meliponids, the level of heterozygosity for MDH is high, as shown comparatively in the present discussion in relation to *Drosophila*, *Philaenus*, *Gryllus* and *Magicicada*. Data obtained by other authors agree with ours. Contel (1980) reported values of heterozygosity for MDH of 0.17 and 0.075 for *Partamona sp.* and *Scaura latitarsis*, respectively. Intralocus MDH variability values for *P. droryana* varied from 0.00 to 0.711, with a mean of 0.240 ± 0.1064 .

Table 1 - Statistical test carried out to ascertain the agreement between observed and expected genotypic proportions in the progenies of informative crosses for MDH. Data for three species are presented: *A. M. marginata marginata*; *B. P. droyana*; *C. P. (Paratamona) helleri*. The χ^2 calculated with the continuity correction of Yates is indicated by the c index (DF = 1).

Species	Cross ♀ x ♂	No. of Nests	Progeny							
			Females			Males				
			Genotypes	Obs	χ^2	P	Genotypes	Obs.	χ^2	P
A	MDHI*1/MDHI*2 x MDHI*2	2	MDHI*2/MDHI*2	27	0.276	n/s	-	-	-	-
			MDHI*1/MDHI*2	31						
B	MDHI*2/MDHI*3 x MDHI*3	2	MDHI*3/MDHI*3	13	0.727	n/s	-	-	-	-
			MDHI*2/MDHI*3	19						
	MDHI*2/MDHI*3 x MDHI*2	1	MDHI*2/MDHI*2	6	1.126 _c	n/s	-	-	-	-
			MDHI*2/MDHI*3	2						
C	MDHI*1/MDHI*3 x MDHI*1	1	MDHI*1/MDHI*1	1	10.286	<0.01	MDHI*1	3	5.40	< 0.05
			MDHI*1/MDHI*3	13			MDHI*3	12		
	MDHI*1/MDHI*2 x MDHI*1	1	MDHI*1,MDHI*1	8	0.222	n/s	-	-	-	-
			MDHI*1/MDHI*2	10						

Table II - Statistical test performed to ascertain the agreement between observed and expected genotypic proportions in the progenies of informative crosses for phosphoglucomutase in *P. droyana*. The χ^2 calculated with the continuity correction of Yates is indicated by the *c* index (DF = 1).

Cross ♀ x ♂	No. of Nests	Progeny							
		Females			Males				
		Genotypes	Obs	χ^2	P	Genotypes	Obs.	χ^2	P
PGM*1/PGM*2 x PGM*2	7	PGM*2/PGM*2	39	0.013	s/n	PGM*1	12	0.154	n/s
		PGM*1/PGM*2	40			PGM*2	14		
PGM*1/PGM*2 x PGM*1	2	PGM*1/PGM*1	12	2.000	s/n	PGM*1	3	0.126 ^c	n/s
		PGM*1/PGM*2	20			PGM*2	5		

Analysis of the genotypic proportions in the progenies of segregant crosses (Tables I and II) revealed statistical significance only for the MDH locus of individuals from a *P. droryana* hive originating from Londrina, Paraná (Table IB) for which the observed genotypic ratios did not agree with those expected on the basis of Mendelian law. The χ^2 values were significant both for females ($\chi^2 = 10.286$; $P < 0.01$) and males ($\chi^2 = 5.40$; $P < 0.05$).

On the basis of the type of crossing proposed for the hive in question ($MDH1*1/MDH1*3 \times MDH1*1$), the expected ratios for the descendants would be 1:1 $MDH1*1$ and $MDH1*3$ males, and 1:1 $MDH1*1/MDH1*1$ and $MDH1*1/MDH1*3$ females also. However, the ratios observed were 1:4 for males and 1:13 for females. Since only one $MDH1*1/MDH1*1$ female was detected, we may postulate the occurrence of experimental error. Thus, a $MDH1*1$ male, for example, may have been computed as a female. This possibility, however, can be ruled out because the morphological distinction of males from females is based on the observation of three easily distinguishable traits. Furthermore, even if an error of this type had occurred, the presence of $MDH1*1$ and $MDH1*3$ males in the progeny implies that the queen's genotype is $MDH1*1/MDH1*3$ as proposed. Thus, if we try to explain these results on the assumption that an $MDH1*1$ male was computed as a female, we shall also have to assume that all the $MDH1*1$ males of the hive in question were produced by workers. If we assume this hypothesis to be true, we would have to invalidate the crossing under consideration and propose that the parental crossing was between an $MDH1*3/MDH1*3$ queen and an $MDH1*1$ drone and that the males produced by workers amounted to 20%. Furthermore, for this hypothesis to be true, we would also have to assume that the workers which supposedly produced only $MDH1*1$ males were not descendants of this crossing ($MDH1*3/MDH1*3 \times MDH1*1$).

Terada (1980), in a study of the developmental pattern of the ovaries of *P. droryana* workers, observed that when the queen is present in the hive, the great majority of the workers's eggs are of the nutrient type, which do not give origin to males. It is only in the absence of the queen that functional eggs seem to develop in workers. This opinion, however, is not shared by Machado *et al.* (1984) who, on the basis of studies of the genetic control of NAD-dependent malate dehydrogenase in *P. drodryana*, concluded that 84.23% of the males are sons of the queen, and 15.77% are sons of workers.

Two other hypotheses could be advanced to explain the discrepancy between the genotypic proportions observed and expected for the *P. drodryana* hive originating from Londrina: 1) the coexistence of more than one egg-laying queen in a single nest, and 2) the occurrence of multiple fertilizations of the egg-laying queen. The segregations observed in three different *P. droryana* hives both for MDH and PGM suggest that at least one of these hypotheses is true.

Studies by Kerr and Krause (1950) and by Contel and Kerr (1976) have shown that *Melipona* queens are fertilized only once and by a single male. However, the data obtained in the present study indicate that, at least for *P. droryana*, the queen may be fertilized by more than one male.

The fact that, out of eleven samples from one hive, three MDH phenotypes were observed among females (Figure 2) and that the analysis of fifty-three pupae from a single hive for PGM revealed no less than five distinct phenotypes among workers (four of which are shown in Figure 3), shows that the observed segregation did not agree with the expected one if we assume the queens to have been fertilized by a single male.

Two additional considerations deserve to be made:

1. The different phenotypes were observed in samples collected on a single occasion, thus eliminating the possibility of an exchange of queen in the hive. Furthermore, the experiments were replicated in all cases;

2. In the hives in which segregations differing from the expected were observed, the samples were collected from a single comb.

Wille (1983), in a review on the biology of meliponids pointed out that in bee species the fertilization of the queen generally occurs during the mating flight. However, it may occur that, as a result of disturbances in the nests, males will mate with inseminated queens unable to fly.

If we add to this fact the observation that monogyny exists in most hymenopteran species (Forsyth, 1980) and that in meliponid hives there is only one egg-laying queen, even though some virgin queens may also be present (Contel, 1972; Del Lama, 1977), we may consider more plausible the hypothesis of the occurrence of multiple fertilizations in the hives for which we obtained discrepant results. It is difficult to accept the possibility that two egg-laying queens, in addition to coexisting in a single hive, will also lay eggs in the same comb.

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RESUMO

De nove espécies estudadas para PGM, duas apresentaram variação genética: *Plebeia droryana* e *Scaptotrigona bipunctata*. O polimorfismo detectado em *P. droryana* é controlado geneticamente por três alelos, enquanto que o polimorfismo de *S. bipunctata* é controlado por apenas dois alelos de um loco. Evidências experimentais indicam que, nestes organismos, a estrutura da PGM é monomérica. Das dez espécies estudadas para a MDH, três foram polimórficas: *Melipona marginata marginata*, *Partamona (Partamona) helleri* e *P. droryana*. Dois alelos de um loco controlam geneticamente o polimorfismo da MDH em *M.m. marginata* e *P. (P.) helleri*, enquanto que a variabilidade da MDH observada em *P. droryana* é regulada por três alelos em um loco. A análise das proporções genotípicas para a MDH em progênes de cruzamentos segregantes de *P. droryana* revelou significância estatística. Duas hipóteses foram levantadas para explicar esta discrepância: a coexistência de mais que uma rainha poedeira em um único ninho e/ou a ocorrência de fertilizações múltiplas da rainha poedeira de um ninho.

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