



Prediction of social structure and genetic relatedness in colonies of the facultative polygynous stingless bee *Melipona bicolor* (Hymenoptera, Apidae)

Evelyze Pinheiro dos Reis, Lucio Antonio de Oliveira Campos and Mara Garcia Tavares

Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Abstract

Stingless bee colonies typically consist of one single-mated mother queen and her worker offspring. The stingless bee *Melipona bicolor* (Hymenoptera: Apidae) shows facultative polygyny, which makes this species particularly suitable for testing theoretical expectations concerning social behavior. In this study, we investigated the social structure and genetic relatedness among workers from eight natural and six manipulated colonies of *M. bicolor* over a period of one year. The populations of *M. bicolor* contained monogynous and polygynous colonies. The estimated genetic relatedness among workers from monogynous and polygynous colonies was 0.75 ± 0.12 and 0.53 ± 0.16 (mean \pm SEM), respectively. Although the parental genotypes had significant effects on genetic relatedness in monogynous and polygynous colonies, polygyny markedly decreased the relatedness among nestmate workers. Our findings also demonstrate that polygyny in *M. bicolor* may arise from the adoption of related or unrelated queens.

Key words: *Melipona*, microsatellites, polygyny, queen number, social structure.

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Introduction

Highly eusocial bees vary greatly in their social structure, with some species typically consisting of one single-mated mother queen and her worker offspring (Peters *et al.*, 1999; Strassmann, 2001). In this case, the queen lays most of the eggs and the sterile workers increase their overall fitness by helping to rear the queens offspring (Hamilton, 1964, 1972). This social organization results in a high degree of relatedness among nestmates. In contrast, other species form colonies with multiple laying queens or multiple mated queens. Polygyny and/or polyandry affect genetic diversity within the colony, decrease the average progeny relatedness, promote interactions among individuals with different levels of relatedness and generate within-colony genetic conflicts (Bourke and Franks, 1995; Keller and Chapuisat, 1999). Superseding of the queen and the transitional stages of colonies (one or multiple queens in different periods of the year, depending on the colony) can also change the social structure of colonies to a more complex one (Herbers and Stuart, 1996; Hastings *et al.*, 1998).

Several studies have demonstrated that queens in polygynous colonies may be completely unrelated or full sisters (Seppa, 1996; Banschbach and Herbers, 1996; Satoh *et al.*, 1997; Goodisman and Ross, 1998; Hastings *et al.*, 1998; Pedersen and Boomsma, 1999; Heinze and Keller,

2000). Consequently, the average genetic relatedness among nestmates in polygynous species can remain relatively close to the value of 0.75 expected with a single once-mated queen (Queller *et al.*, 1988; Strassmann *et al.*, 1991; Herbers, 1993; Rosengren *et al.*, 1993; Crozier and Pamilo, 1996; Bourke *et al.*, 1997; Fournier *et al.*, 2002).

Stingless bee colonies typically consist of one single-mated mother queen and her worker offspring (Carmargo, 1972; Peters *et al.*, 1999; Strassmann, 2001; Palmer *et al.*, 2002; Tóth *et al.*, 2003). These two castes are morphologically distinct and the workers are unable to mate, although in many species they can produce haploid eggs (Sakagami *et al.*, 1963). However, the process of worker egg laying is very diverse among different species and the frequency of males that are sons of workers varies from species to species (Sommeijer and van Buren, 1992; Koedam *et al.*, 1996; Sommeijer *et al.*, 1999; Cruz-Landim, 2000; Drumond *et al.*, 2000; Tóth *et al.*, 2004). For example, *Frieseomelitta silvestri* workers are physiologically incapable of laying eggs (Boleli *et al.*, 2000) while in *Melipona scutellaris* and *M. favosa*, 22.88% and 94.5%, respectively, of the males are sons of workers (Sommeijer *et al.*, 1999; Chinh *et al.*, 2003; Alves *et al.*, 2009).

In some stingless bees, transitory periods of polygyny have been described and are generally associated with queen replacement. Witter and Wittmann (1997) described a polygynic colony in *Plebeia wittmanni* in which a new queen started oviposition while the older one was still alive and laying eggs. Carvalho-Zilse and Kerr (2004) reported a

similar case for *M. scutellaris*, and recently Alves *et al.* (2010) reported on a polygynic colony of *M. quadrifasciata* in which eight egg-laying queens coexisted in a colony for ca. four months.

Melipona bicolor (Hymenoptera: Apidae) is a stingless bee species that naturally displays facultative and long-lasting polygyny, with the number of queens varying among populations and even among colonies of the same population. Some colonies are headed by a single queen, whereas others have more than one queen living together for considerable periods of time (Cepeda, 2006; Velthuis *et al.*, 2006). Velthuis *et al.* (2001) observed up to five physogastric queens laying eggs in a colony of *M. bicolor* and noted that workers did not show preference or aggressiveness to foreign queens.

In *M. bicolor* polygynous colonies, queens do not show antagonism, territoriality, aggressiveness or competition (Bego, 1989) and workers do not distinguish among the physogastric queens, to which they have different degrees of relatedness (Alonso *et al.*, 1998). Additionally, more than one queen is frequently seen inspecting the provisioning process (Velthuis *et al.*, 2001). During this process, the workers construct and mass provision the cells with a mixture of regurgitated nectar and pollen. Thereafter, one of the queens lay her eggs on top of this liquid and, finally, the workers seal the cells (Sakagami, 1982). Within each cell, the offspring develops with no further interference from its kin. Polygynous colonies are not larger or more productive than monogynous ones (Velthuis *et al.*, 2006). Like other stingless bees, *M. bicolor* colonies are long lived, persisting perhaps for decades, while queens may live for 1-3 years (Velthuis *et al.*, 2006).

The variation in social structure of *M. bicolor* affects the genetic diversity within the colony, the relatedness among nestmates, and the architecture of potential kin-conflicts and kin cooperation (Bourke and Franks, 1995; Crozier and Pamilo, 1996). Hence, understanding the dynamics of variation in queen number is fundamental for a better understanding of the social evolution in this species. Data on the dynamics of queen number and on variation in social structure over time are also needed to obtain a clearer picture of how complex social structures appear and are maintained.

Since the number of queens and their genetic relatedness, as well as their relative reproductive success, can vary among colonies, we investigated the social structure and genetic relatedness among workers from 14 colonies of *M. bicolor* over one year in order to test the theoretical expectations concerning social behavior.

Materials and Methods

Samples

Eight natural colonies (six from Caeté and two from Cataguases in the southeastern Brazilian state of Minas

Gerais, MG) and six manipulated colonies of *M. bicolor* (maintained in artificial hives at the Central Apiary of the Universidade Federal de Viçosa, Viçosa, MG) were sampled monthly from March 2007 to January 2008. Natural colonies were maintained in their original shelter and the workers were sampled directly from the colony entrance, without opening the colony. In contrast, manipulated colonies were opened to collect the workers. In these cases, manipulation was minimized to avoid alteration in the organization of the colony. The social structure of the colonies was monitored over a one year period by analyzing ten workers/colony that were collected in March, May, June, September and November 2007 and January 2008 (total of 60 workers/colony). Males were not analyzed because they generally represent worker offspring.

DNA extraction and microsatellite analysis

DNA was extracted according to the protocol recommended by Waldschmidt *et al.* (1997), using adult workers. Four microsatellite loci (Mbi11, 201, 233 and 278) were analyzed using the amplification conditions described by Peters *et al.* (1998). The polymerase chain reaction (PCR) amplifications were done in reaction volumes of 10 μ L containing 12.5 ng of genomic DNA, 1X *Taq* PCR buffer, 1 U *Taq* DNA polymerase (Promega), 0.5 μ M of each forward and reverse primer, 0.1 mM dNTP and 1.5 mM $MgCl_2$. The conditions for the PCR were the following: 3 min at 94 °C followed by 40 cycles of 30 s at 92 °C, 1 min at the specific primer pairing temperature and 30 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were separated on 8% denaturing polyacrylamide gels and visualized by staining with 0.2% silver nitrate.

Genetic data analysis

Since the aim of this study was to analyze the dynamics of queen numbers and variation in the social structure over time, the queens were not collected or genotyped. Instead, assuming that queens mated with a single male (Peters *et al.*, 1999; Strassmann, 2001), queen and fathering male genotypes, as well as the social structure (polygyny or monogyny) of each colony, were determined indirectly from worker genotypes. This allowed us to infer the minimum number of queens in each colony. When all workers were heterozygous (A/B, for example) for a determined locus, alleles from queens and the males that mated with them could not be determined with precision. In these cases, queen and male genotypes were considered as A/A or B/B and B or A, respectively.

The non-detection error due to paternal males displaying identical genotypes by chance was estimated for each population as:

$$\Pi (\sum q_i^2)_j$$

where q_i denotes the allele frequencies at each of j loci (Foster *et al.*, 1999).

The monthly genetic relatedness among nestmate workers, the social structure of colonies and the genetic relatedness among queens in the polygynous colonies were estimated according to Queller and Goodnight (1989) by using the software Relatedness 4.2 (Goodnight and Queller, 1994). Colonies were equally weighted and the standard error was obtained by jackknifing over loci. Relatedness and the genetic relationships among queens from polygynous colonies were also checked by inspecting their multilocus genotypes or the inferred multilocus genotypes of the original pair (the original queen and her mate) and that of the new queen, in cases of queen replacement or queen adoption.

The allelic frequencies of each locus and the observed (H_o) and expected (H_e) heterozygosities (Table S1 – Supplementary Online Material) were calculated using PopGene version 1.32 (Yeh *et al.*, 1999).

Results

Direct observation of worker genotypes showed that eight of the 14 colonies analyzed (Cataguases 1, 2, Caeté 2, 3, 7, Viçosa 916, 921 and 934) had genotypes consistent with a single queen mated to a single male. On the other hand, colonies Caeté 1, 5 and 6, and Viçosa 814, 905 and 915 were polygynous. The number of queens in each of these colonies was two, except for Caeté 5, the workers of which had genotypes consistent with three queens. In the

manipulated colony Viçosa 915, the two queens were seen throughout the period of sampling and relatedness estimates confirmed their polygynic status. In the other manipulated colonies (Viçosa 814 and 905), there was no visual confirmation of the presence of multiple queens. This lack of visual confirmation partly reflected the minimal manipulation of the colonies in order to avoid altering their organization and the fact that the queens generally hide during manipulation. The presence of multiple queens in natural colonies could not be confirmed visually because the colonies were not opened during sampling.

The estimated genetic relatedness among workers from monogynous and polygynous colonies was 0.75 ± 0.12 (mean \pm SEM; range: 0.49-0.96) and 0.53 ± 0.16 (mean \pm SEM; range: 0.21-0.90), respectively (Table 1). The high genetic relatedness ($r = 0.90 \pm 0.15$) detected among workers from the polygynous colony Caeté 1 in September resulted from the sampling of workers from a single matriline. The effects of non-detection error at the population level, which can lead to an overestimation of relatedness (Boomsma and Ratnieks, 1996; Foster *et al.*, 1999), were relatively low (0.08; range: 0.017 to 0.22) and exerted only a minor additional impact on the mean nestmate relatedness.

The estimated relatedness among queens from polygynous colonies revealed that queens from colonies Viçosa 814, Viçosa 905, Caeté 6 and two out of the three queens

Table 1 - Estimated genetic relatedness ($r \pm$ SE) among *Melipona bicolor* workers.

Status	Colonies	Relatedness						
		March	May	July	September	November	January	Mean
Monogynous colonies								
	Caeté 2	0.62 \pm 0.21	0.69 \pm 0.19	0.63 \pm 0.23	0.60 \pm 0.22	0.66 \pm 0.18	0.66 \pm 0.21	0.64 \pm 0.03
	Caeté 3	0.89 \pm 0.10	0.86 \pm 0.11	0.88 \pm 0.12	0.89 \pm 0.10	0.88 \pm 0.11	0.89 \pm 0.10	0.88 \pm 0.01
	Caeté 7	-	0.72 \pm 0.12	0.76 \pm 0.13	0.86 \pm 0.13	0.96 \pm 0.04	0.87 \pm 0.11	0.83 \pm 0.09
	Cataguases 1	0.82 \pm 0.09	0.69 \pm 0.14	0.69 \pm 0.07	0.68 \pm 0.16	0.79 \pm 0.14	0.70 \pm 0.13	0.73 \pm 0.06
	Cataguases 2	0.75 \pm 0.08	0.74 \pm 0.07	0.73 \pm 0.11	0.85 \pm 0.08	0.83 \pm 0.13	0.74 \pm 0.08	0.77 \pm 0.05
	Viçosa 916	0.52 \pm 0.03	0.49 \pm 0.06	0.55 \pm 0.07	0.53 \pm 0.04	0.58 \pm 0.08	0.50 \pm 0.11	0.53 \pm 0.03
	Viçosa 921	0.66 \pm 0.27	0.66 \pm 0.31	0.85 \pm 0.11	0.86 \pm 0.11	0.85 \pm 0.13	0.86 \pm 0.12	0.86 \pm 0.005
	Viçosa 934	0.77 \pm 0.14	0.72 \pm 0.10	0.74 \pm 0.08	0.71 \pm 0.12	0.73 \pm 0.15	0.70 \pm 0.14	0.73 \pm 0.02
	Mean							0.75 \pm 0.12
Polygynous colonies								
	Caeté 1	0.53 \pm 0.20	0.41 \pm 0.20	0.32 \pm 0.05	0.90 \pm 0.15	0.23 \pm 0.14	0.33 \pm 0.09	0.45 \pm 0.24
	Caeté 5	0.39 \pm 0.21	0.43 \pm 0.21	0.36 \pm 0.20	0.59 \pm 0.13	0.45 \pm 0.22	0.36 \pm 0.23	0.43 \pm 0.08
	Caeté 6	0.80 \pm 0.05	0.64 \pm 0.16	0.64 \pm 0.15	0.59 \pm 0.15	0.60 \pm 0.18	0.67 \pm 0.12	0.66 \pm 0.07
	Viçosa 814	0.72 \pm 0.08	0.65 \pm 0.13	0.79 \pm 0.09	0.79 \pm 0.09	0.66 \pm 0.13	0.75 \pm 0.11	0.73 \pm 0.06
	Viçosa 905	0.70 \pm 0.11	0.46 \pm 0.11	0.46 \pm 0.13	0.60 \pm 0.10	0.68 \pm 0.05	0.72 \pm 0.15	0.60 \pm 0.11
	Viçosa 915	0.24 \pm 0.06	0.23 \pm 0.07	0.24 \pm 0.09	0.48 \pm 0.05	0.35 \pm 0.10	0.21 \pm 0.15	0.29 \pm 0.10
	Mean							0.53 \pm 0.16

from colony Caeté 5 were close relatives ($r = 0.76 \pm 0.008$, 0.56 ± 0.009 , 0.74 ± 0.008 and 0.81 ± 0.15 , respectively), while the third queen of colony Caeté 5 was less related to the others ($r = 0.32 \pm 0.03$). Similarly, queens from colonies Caeté 1 and Viçosa 915 were also not closely related to each other ($r = -0.08 \pm 0.04$ and 0.29 ± 0.02 , respectively).

At the colony level, the social structure was very stable throughout the study (12 consecutive months): queen numbers did not change in 12 out of the 14 analyzed colonies. A case of queen replacement was detected in colony Viçosa 921 in July, and a case of queen adoption was detected in colony Caeté 6 in May (in March a single queen was detected in this colony). The two queens detected in colony Caeté 6 in May persisted in the colony until the end of the sampling period; this finding strengthened the hypothesis that polygyny is stable in *M. bicolor*.

The allelic variation and heterozygosity indices observed in the 14 colonies, the inferred genotypes of queens and drones and the number of workers attributed to each queen in the polygynous colonies are summarized in Tables S1, S2 and S3, respectively, of the Supplementary Material.

Discussion

The genetic analysis of *M. bicolor* workers confirmed that monogynous and polygynous colonies are widespread in natural (Caeté) and manipulated (Viçosa) populations. In monogynous colonies, the progeny had a maximum of three alleles at each locus this being consistent with one paternal and up to two maternal alleles; this finding also confirmed that in these colonies workers are the progeny of single-mated queens.

Although the estimated genetic relatedness among workers from monogynous and polygynous colonies varied considerably throughout the year and from colony to colony, the average relatedness estimates for these colonies (0.75 and 0.53, respectively) were in good agreement with theoretical expectations for these social structures (Bourke and Franks, 1995). Additionally, the average relatedness estimated for monogynous colonies was very similar to the mean of 0.739 reported by Peters *et al.* (1999) for 12 species of single-queen stingless bees.

In the case of monogynous colonies, the results demonstrated that parental genotypes have a significant effect on relatedness in this species. For example, workers from the monogynous colony Viçosa 916 had an estimated genetic relatedness ranging from 0.49 to 0.58. This probably occurred because the queen was heterozygous for all loci analyzed and the paternal alleles were different from the queen's alleles at all of these loci. Similar results were observed for colony Caeté 2. Consequently, in these two colonies the estimated genetic relatedness among workers differed considerable from the expected value of 0.75 for full sisters.

Our results also demonstrated that the estimated genetic relatedness among workers from the monogynous colony Viçosa 921 was relatively low in March and May compared to the other months. In this case, the workers were found to be the progeny of two queens. In May, however, the progeny of one of these queens decreased substantially and in the subsequent months the workers sampled represented the progeny of a single queen. This finding suggested that an older queen was replaced by a new one, with the samples from March and May representing the transitional phase of this otherwise monogynous colony. Comparisons between the inferred multilocus genotypes of the original pair (the original queen and her mate) and that of the new queen and the genetic relatedness between queens ($r = 0.86 \pm 0.14$) revealed that both queens were very closely related (probably mother and daughter).

Analysis of the worker genotypes from colony Caeté 5 showed that they were more related to each other than were workers from Viçosa 915. This occurred because two of the three queens from colony Caeté 5 were closely related, while the two queens from Viçosa 915 were unrelated. Worker genotypes and the inferred queen/male genotypes further confirmed that the queens from colony Caeté 1 were unrelated. In these cases, the unrelated queens may have been adopted secondarily. In contrast, the two queens present in each of the colonies Viçosa 814, Viçosa 905 and Caeté 6 were full sisters.

These findings support the view that polygyny in *M. bicolor* may arise by two mechanisms. First, since, as in other *Melipona* species, *M. bicolor* queens are produced all year long and may persist in the colony, polygyny may arise from the adoption of a related queen (mother-daughter or full sisters), thus confirming observations of Velthuis *et al.* (2006). In such cases, variation in the number of queens may have no detectable effect on relatedness. In other cases, however, unrelated queens may be secondarily adopted by established colonies of *M. bicolor*, thereby diminishing the relatedness among nestmate workers. Overall, polygyny markedly reduced the relatedness in *M. bicolor* (from 0.75 to 0.53).

The limited variation in the number of queens in monogynous and polygynous colonies over a one year period agreed with the common view that queen replacement is rare in stingless bee colonies (queen replacement was detected in only one of the 14 analyzed colonies here) and that queens have long life spans. Queens of *Melipona compressipes* and *M. scutellaris*, for example, have a maximum longevity of 84 months (7 years) (Carvalho-Zilse and Kerr, 2004). This reduced turnover of queens could help to maintain the relatedness among nestmate workers stable over time.

The single-mating behavior of *M. bicolor* means that workers from monogynous colonies are highly related and this seems to be a key factor in the evolution of eusociality and the maintenance of their altruistic behavior (Cole,

1983; Boomsma, 2007). In contrast, in polygynous colonies, the degree of relatedness among workers will decrease according to the queen-queen relatedness.

Kin selection theory predicts conflicts between queens and workers over male production in monandrous/monogynous colonies of *M. bicolor* because workers are more related to their own eggs ($r = 0.5$) and to other workers eggs ($r = 0.375$) than they are to the eggs of the queens ($r = 0.25$) (Ratnieks, 1988). In these cases, workers could attempt to monopolize male production. However, in polyandrous/polygynous colonies in which workers are on average more related to sons of the queens ($r = 0.25$) than to the sons of other workers ($r = 0.125$) they may “prefer” the queen-laid eggs, as already verified for some polyandrous species such as *Apis mellifera* (Ratnieks, 1988; Ratnieks and Visscher, 1989) and *Vespula vulgaris* (Foster and Ratnieks, 2001).

Evidence for queen-worker conflict can be found in the cell provisioning and oviposition process of most stingless bee species. *M. bicolor* queens, in particular, undertake elaborate, ritualized interactions with workers before and during egg-laying (Velthuis *et al.*, 2006). Additionally, as with many stingless bees, *M. bicolor* workers in monogynous and polygynous colonies have been observed to lay reproductive and/or trophic eggs (Koedam *et al.*, 2001). These authors observed that after laying a trophic egg, the worker generally leaves the cell, giving the queen the opportunity of eating this egg. In the case of a reproductive egg, workers usually close the cell immediately after oviposition. Competing reproductive workers, however, frequently eat and replace this egg by their own (Velthuis *et al.*, 2002; Koedam *et al.*, 2007).

In polygynous colonies with highly related queens (such as observed in several colonies in this study), the reasonably high levels of relatedness among workers could lower the costs of sharing reproduction (Giraud *et al.*, 2001). In this case, kin selection may inhibit the workers' ability to become reproductive since they can gain greater inclusive fitness by functioning as helpers of close relatives (Hamilton, 1964; Queller and Strassmann, 1998). On the other hand, in polygynous colonies with queens that are not closely related, workers would lay most of the male progeny because of the lower intra-colony relatedness (Koedam *et al.*, 2007). Accordingly, in a polygynous colony of *M. bicolor* containing three unrelated queens (two of them introduced experimentally), 27%-82% of the males were workers sons. In this colony, workers replaced reproductive eggs laid by other workers with their own, but they tended to avoid eating the queen's eggs; this behavior was considered a form of policing by reproductive workers (Koedam *et al.*, 2007).

In conclusion, the results described here provide additional insights into the social structure and relatedness in *M. bicolor*. However, further research is needed in order to clarify the conflict over male parentage in monogynous and

polygynous colonies, and to address other aspects related to worker oviposition, such as worker policing by egg eating. Such studies will lead to a better understanding of the development of social behavior in *Melipona*.

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Supplementary Material

The following online material is available for this article:

Table S1 – Allelic frequencies and observed (H_o) and expected (H_e) heterozygosities in colonies of *Melipona bicolor*.

Table S2 – Inferred genotypes of queens and drones from monogynous and polygynous colonies of *Melipona bicolor*.

Table S3 – Monthly contribution of queens to progeny constitution in polygynous colonies of *Melipona bicolor*.

This material is available as part of the online article from <http://www.scielo.br/gmb>.

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