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Research article

Parental analysis using RAPD markers in the ant *Colobopsis nipponicus*: a test of RAPD markers for estimating reproductive structure within social insect colonies

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Key words: RAPD, DNA polymorphism, parental analysis.

Summary

Previously known parent-offspring relationship for queens and her daughters of the ant *Colobopsis nipponicus* was examined using RAPD markers in order to test the reliability of this molecular technique for estimating the reproductive structure within colonies of social insects. RAPD markers from 20 oligomers successfully clustered the queen with her daughters among an artificially generated polygynous society, even when paternal information was unavailable. When information from both the queen and her sperm was included in the analysis, 20 polymorphic bands seem to be sufficient to cluster correctly the true parents to their offspring. Lack of father's information considerably decreased accuracy of the analysis. Thus, if RAPD markers are to be used to demonstrate parent-offspring relationship between individuals in the field, sperm from the queen's spermatheca should be incorporated in analysis.

Introduction

Reproductive division in a colony is a fundamental characteristic of insects sociality (Wilson, 1971; Hölldobler and Wilson, 1990). Reproductive structure (who reproduces and what is the reproductive output of these) as well as genetic relationship between reproductives and other colony members have a major effect on evolution of sociality (Hamilton, 1964). Thus, for studies of insects sociality, it is especially important to known reproductive structure within a colony (see Keller and Vargo, 1993; Reeve and Ratnieks, 1993). However, it is usually difficult to demonstrate reproductive structure explicitly because a colony may contain multiple reproductives and include worker-produced males (reviewed in Bourke, 1988; Keller and Vargo, 1993).

Several molecular markers have been developed to detect genetic variation among individuals, e.g. allozyme markers and DNA fingerprint. Although these

markers are useful to elucidate genetic relationships between individuals, it is nearly impossible to apply these methods to small insects because the required amount of tissue or genomic DNA is too large to conduct multiple experiments. Polymerase chain reaction (PCR) can amplify specific regions of DNA by a pair of nucleotide primers and may therefore solve this problem. Indeed, several new methods using PCR have been developed to detect individual-level genetic variations (reviewed by Queller et al., 1993).

Random amplified polymorphic DNA (RAPD) is a recently developed molecular technique that amplifies many regions of genomic DNA randomly and gives a multi-locus DNA fingerprinting pattern (Williams et al., 1990). Several studies have shown mendelian inheritances of RAPD markers (Williams et al., 1990; Carlson et al., 1991; Martin et al., 1991; Welsh et al., 1991; Hunt and Page, 1992; Bucci and Menozzi, 1993), and some studies have applied RAPD markers to identify parent-offspring relationship (Riedy et al., 1992; Fondrk et al., 1993; Hadrys et al., 1993; Levitan and Grosberg, 1993). Fondrk et al. (1993) successfully assigned daughter workers of a single honeybee queen to their patriline, whereas Riedy et al. (1992) failed to determine parentage of a child baboon because of excess non-parental bands in RAPD markers. Thus, it has become necessary to test the ability of RAPD markers to identify the reproductive structure within populations with complex social structures, such as ant colonies with polygyny or worker reproduction.

In this paper, I examined reliability of RAPD markers as a tool for estimating genetic relationship within colonies of the ant *Colobopsis nipponicus*, for which reproductive structure have been well studied previously (Hasegawa, 1992, 1994). In addition, by using an artificially generated polygynous colony, I examined the ability of RAPD markers to assign workers from different matrilines to their true matriline, in cases including those in which paternal information is not available.

Materials and methods

Reproductive structure of C. nipponicus

C. nipponicus is an obligately monogynous ant distributed in Japan (Hasegawa, 1992). A previous study using an allozyme marker confirmed that the colony queen mated with a single male and produced all offsprings (including males) within her colony (Hasegawa, 1994). Thus, this species has the simplest reproductive structure within colonies, i.e., monogyny, monoandry and no worker reproduction.

Sampling and DNA preparation

In December 1993, 3 colonies of *C. nipponicus* were collected from a coastal forest near Amatsukominato, Chiba Prefecture, Japan. Each colony contained a single queen, several dozens of workers and many larvae. The queen and 5 workers were selected from each colony, and the workers were preserved in pure ethanol. Each freshly killed queen was dissected under a binocular microscope to collect sperm in her spermatheca. The queen's spermatheca was removed from the body and was

punctured in 5 µl of distiled water. This solution was then frozen at −80 °C until DNA extraction. The residual queen body was preserved in pure ethanol.

The individual queen body and workers were desiccated on filter paper, then grounded by a disposable pestle in 500 µl of an extraction buffer (0.5 % SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.2 mg Proteinase K (Wako), 0.01 mg RNase A [Boehringer, Mannheim]) within a 1.5 ml microcentrifuge tube. Samples were incubated at 55 °C over 3.5 hr. Following 2 phenol and 1 phenol-chloroform extraction, 40 µl of 2 M CH_3COONa was added to the aqueous phase, and DNA was precipitated by 700 µl of pure 2-propanol. The sample was incubated at −80 °C or 30 min and then centrifuged for 15 min at 11200 × g and 4 °C. The DNA pellet was rinsed twice with chilled 70 % ethanol and resuspended in 20 µl of TE buffer (1 mM EDTA, 10 mM Tris-HCl (pH 8.0)). The DNA was quantified with a fluorometer (HITACHI U-3210) and diluted to 5 ng/µl with double distiled water.

RAPD reaction

Genomic DNA was amplified using the PCR technique on a PTC 100 thermal cycler (MJ research Inc.) and a 10 bp long primer of random nucleotide sequence (Operon Technologies Inc.). A total of 20 primers was used in this study. Each PCR reaction (5 µl) contain 5 ng template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $mgCl_2$, 0.001 % gelatin, 6pmol of one of 20 primers, and 0.2 unit Taq DNA polymerase (Amplitaq, Perkin Elmer). Amplification procedures consisted of 45 cycles of 1 min at 94 °C, 1 min at 35 °C, a 0.3 °C/sec temperature transition to 72 °C and 2 min at 72 °C. PCR products were separared by electrophoresis in 8 % polyacrylamide gel with 1 × TBE, and were visualized by a silver staining (Tegelström, 1986).

Ability of RAPD markers to discriminate multiple matrilines

Reliability of RAPD markers was examined first. The total DNA of the queen of Colony 1 was amplified in 5 reaction tubes by the same cycle. This procedure was repeated, and the 10 products were screened on the same gel to confirm reproductivility of the same pattern.

For each of the 3 colonies, total DNA of every 5 workers, their queen and the queen's sperm were amplified individually using each of the 20 primers. The PCR products from the analysis of 1 primer were screened on the same gel. This yielded 20 gels with 21 RAPD band patterns on each gel (3 colonies, each comprising a queen, her sperm, and her 5 workers). Any RAPD band with a size larger than 1 kbp was omitted from the analysis because such large DNA segments may not be amplified consistently.

Non-parental RAPD bands, i.e., band that are present in the offspring but not in its parents, were identified in the offspring's patterns. The paternal bands that were present in sperm pattern but not in the daughter's pattern were also checked. All paternal RAPD bands will be present in each offspring because of male haploidy and the single mating of *C. nipponicus* queens (Hasegawa, 1994). Thus,

such paternal bands do not correctly indicate parent-offspring relationship and can be regarded as non-parental RAPD bands. Although the both type of non-parental RAPD bands, hereafter called nonspecific bands, may have a negative effect on a parental analysis (Riedy et al., 1992), I chose to include them into the analysis because nonspecifity of a band cannot be previously determined in field samples with unknown social structure.

To test the ability of RAPD markers to discriminate workers belonging to a single matriline from workers to other matrilines, a set of 3 monogynous colonies was regarded as a data set of a polygynous colony. To estimate parentage of workers, I used 2 different sets of parental information: (1) both queen and sperm patterns and (2) only the queen pattern. The second set of parental information was included because it resembles a realistic situation – only DNA from the queen and her offspring is available.

RAPD reaction yielded some nonspecific bands and these do not follow mendelian inheritance (Riedy et al., 1992; Fondrk et al., 1993; Levitan and Grosberg, 1993). Thus, I did not use diagnostic methods which are based on the assumption of mendelian inheritance of markers because these methods are sensitive to existence of nonspecific bands in the data set. Instead, I applied a multivariate cluster analysis (Ward's linkage method) of a data set comprising the presence or absence of RAPD bands. This method has been recommended by Fondrk et al. (1993) for parental analysis using RAPD markers because it considers the covariance between individuals within and between groups (Sneath and Sokal, 1973) and thus focuses on shared characters rather than on unique or rare characters. Therefore, this method would minimize negative effects of nonspecific bands in the parental analysis.

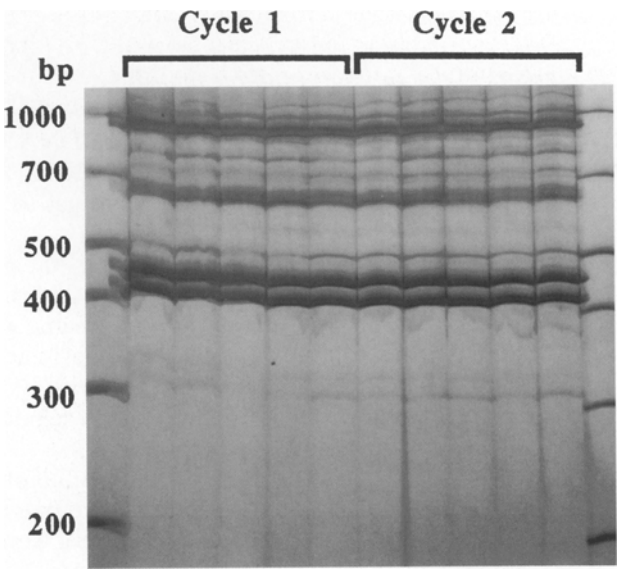

Figure 1. Ten replicated amplification products of RAPD reaction for total DNA of a single individual

In the first analysis, band patterns from the queen and her sperm was combined to generate a parent's band pattern, whereas in the second analysis only the queen's band pattern was used. Only bands with presence/absence polymorphism among parents (queens) were used because monomorphic bands have no information for the worker clustering. The presence or absence of these bands was entered into a data matrix which formed the basis for multivariate clustering of the 18 analytical units (3 parents [queens] and 15 workers).

Number of polymorphic bands required for correct analysis

To examine the required number of polymorphic bands for the reliable analysis, I resambled 5, 10, 15, 20, 25, 30, 35, and 40 bands 100 times from the all polymorphic bands (with replacement). Then, the clustering was conducted on each resumpled data set, and the percentage of correct clusters was calculated for each band number. This procedure gives an estimate of the minimum required number of polymorphic bands for the parental analysis.

Results

Figure 1 shows 10 independent products of RAPD reaction for total DNA of the same individual. There is no difference in the band pattern between cycles or between tubes. Thus, I concluded that there is no unreliable amplification due to nonspecific priming of the primer and that RAPD markers are reliable for detecting individual genetic variability. Figure 2 indicates an example of polymorphic band.

Table 1 shows summary of the amplification by each primer. Two of the 20 primers (OPA06 and OPA15) failed to amplify any DNA fragments due to unknown reasons. The other 18 primers produced a total of 217 bands of which 97 bands shows presence/absence polymorphism among the examined individuals. Number of RAPD bands and proportion of polymorphic bands in the amplified bands varied from 5 to 21 and 0 to 100%, respectively. Of the 97 polymorphic bands, 10 were nonspecific. These 10 bands were generated by only 4 primers. In the

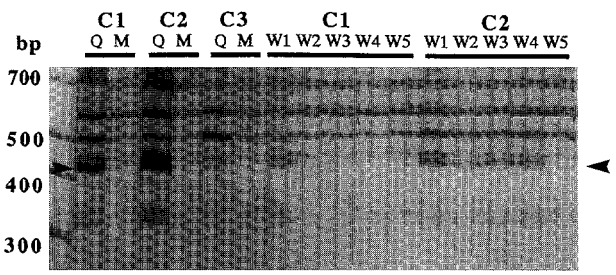

Figure 2. An example of a polymorphic band. This band (shown by the arrow) correctly represents the parent-offspring relationship

Table 1. Summary of the result of DNA amplification by 20 primers

Primers	Number of bands			% of polymorphism
	amplified	polymorphic	non-specific	
OPA1	7	2	0	28.6
OPA2	14	8	0	57.1
OPA3	17	8	0	47.1
OPA4	10	2	0	20.0
OPA5	11	9	3	81.9
OPA6	0	0	0	–
OPA7	9	6	0	66.6
OPA8	9	6	0	66.6
OPA9	11	4	0	36.4
OPA10	16	12	0	75.0
OPA11	21	14	4	66.7
OPA12	14	0	0	0.0
OPA13	11	0	0	0.0
OPA14	5	5	1	100.0
OPA15	6	0	0	0.0
OPA16	0	0	0	–
OPA17	14	11	2	78.6
OPA18	17	3	0	17.6
OPA19	11	2	0	18.2
OPA20	14	5	0	35.7
Total	217	97	10	44.7

products of these 4 primers, proportion of polymorphic bands (39/51, 76,4%) are significantly higher than in the products of the other primers (58/166, 34.9%; χ^2 test, $P<0.05$). This suggested that more polymorphic primers tend to amplify non-specific bands.

The number of polymorphic bands was 42 among the 3 parents and 45 among the 3 queens. Ward's linkage method succeeded in clustering the queens together with their five workers regardless of the presence of the paternal informations (Figs. 3, 4). The clustering was correct even when paternal information was lacking. Thus, in spite of the existence of the nonspecific bands, RAPD markers were effective to cluster related individuals.

Figure 5 shows the results of the resampling simulations. When both queen and sperm information was included in the analysis, 20 bands were enough to identify the true parent-offspring relationship every time. However, when only queen information was used, more than 30 bands were required to obtain the correct relationship. In many cases of the latter analysis, about 20 bands were sufficient to cluster the sister workers correctly, but these worker groups were connected with a wrong queen or with another worker group. Thus, the lack of sperm information considerably decreased the ability of RAPD markers to cluster the queens and their offspring.

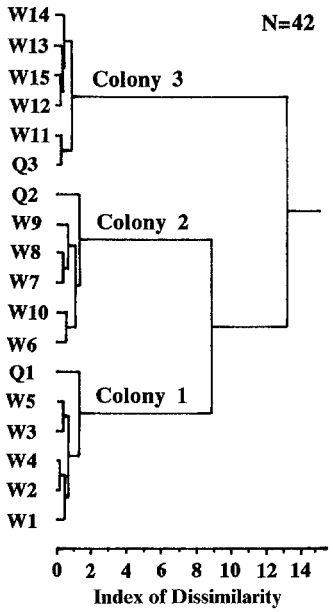

Figure 3. The result of multivariate cluster analysis based on polymorphic parent bands. All workers were correctly clustered with their sisters and true parents

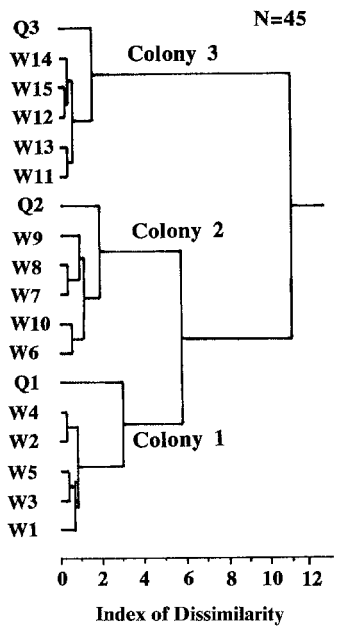

Figure 4. The result of multivariate cluster analysis based on polymorphic queen bands. All workers were correctly clustered with their sisters and true queens

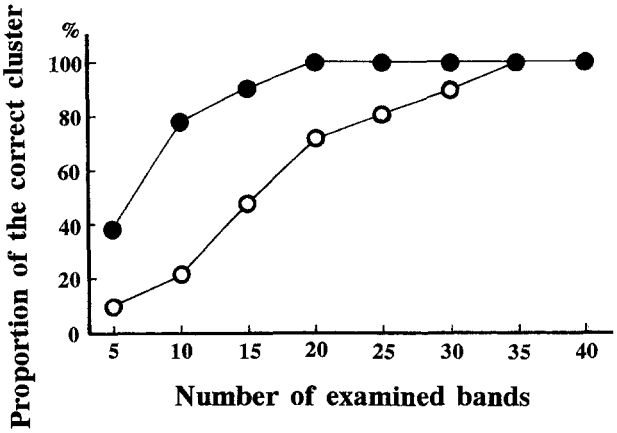

Figure 5. The relationship between the number of bands analysed and success rate in a resampling simulation. Each trial resampled 5, 10, 15, 20, 30, 35, or 40 polymorphic bands from the original data set, and clustered individuals based on the resampled data set. The trials were repeated 100 times. The success rate is the percentage of the correct cluster in 100 trials. Closed circles represent the results based on parent bands. Open circles indicate the results obtained from the analysis including only queen bands

Discussion

RAPD markers can cluster sister workers to their true mother even when paternal information was unavailable. A *C. nipponicus* queen mates with a single male (Hasegawa, 1994), and thus, effect of the lack of paternal information should be minimized. Although the correct clustering was obtained, the lack of paternal imformation considerably decreased the accuracy of the analysis. If the parental analysis is conducted on species with polyandrous queens, the negative effect of lack of paternal information will increase.

A major merit of molecular techniques using PCR is its ability to amplify a small amount of DNA. The analysis of DNA of sperm from the queen's spermatheca is impossible without the PCR technique. The RAPD technique facilitates the amplification of products from a few ng (10^{-9}g) of DNA (Williams et al., 1990), and this study succeeded in amplifying the DNA of the sperm from queen's spermatheca. Therefore, sperm from queen's spermatheca should be used whenever possible in the parental analysis using RAPD markers.

Some RAPD markers did not reflects parent-offspring relationship (Table 1). The presence of such bands, which in some cases occur in large numbers, prevented Riedy et al. (1992) from identifying the mother of a child baboon. Other studies succeeded in assigning offsprings to their parents or patriline by excluding highly polymorphic primers from the analysis (Fondrk et al., 1993; Levitan and Grosberg, 1993). In this study, the appearance of nonspecific bands was limited in the products of 4 primers, and these were more polymorphic than the other ones. Exclusive of products of highly polymorphic primers may increase the accuracy of the analysis. However, I included all polymorphic bands in the analysis because nonspecifity of

a band is usually impossible to determine for obtained RAPD bands of samples from the field. Therefore, in field studies, cluster analysis using RAPD markers should be done with all information. In this study, even when nonspecific bands were included in the analysis, the correct cluster was obtained because Ward's linkage method is relatively insensitive to such rare noise bands. Therefore, RAPD markers can potentially elucidate parent-offspring relationship among individuals from field colonies.

Although the source of the nonspecific amplification was unknown, a possible source may be mitochondrial DNA (mtDNA) carried in the cytoplasm of the sperm. Because mtDNA is relatively shorter (ca. 16 kbp) than genomic DNA, the likelihood of amplification from mtDNA is low. However, this is a possible source of nonspecific bands in RAPD markers. If some bands are amplified from the mtDNA of the sperm, these bands do not appear in offspring bands because of maternal inheretance of mitochondrion. Thus, deterministic analysis methods based on common-sharing of a band on a locus are inadequate for RAPD markers because these methods are sensitive to such nonspecific bands that inevitably appear in the data set.

This study showed that RAPD markers are potentially useful for the analysis of parent-offspring relationships. There would exist, however, some weakness when this method is applied to field samples. As mentioned above, deterministic methods are inadequate, and clusterings are better methods for parent-offspring analysis using RAPD markers. However, cluster analysis nessessarily connects all individuals in the analysis, and it therefore may be problematic for parent-offspring analysis to determine how degree of clustering distance among individuals within a cluster represents the true parent-offspring relationship. For example, when queens in a polygynous colony are closely related, band sharing rate between workers belonging to different matrilines will increase, and thus, the accuracy of cluster analysis will decrease. In *C. nipponicus*, this problem would not arise because queens that inhabit the same tree are not related to each other and queens mate with single males (Hasegawa, 1994). Thus, when RAPD markers are applied to a species with complex social structure, such as polygyny and polyandry, the obtained results should be carefully interpreted.

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