although there was no effect of temperature (16–27°C) on the survival rate during post-diapause development to adult molting (ENDA and IGARASHI, 1988). The deleterious effect of higher temperatures on adult survival rate in pine logs may have been due to greater energy consumption at higher temperatures during the sclerotization and the excavation of the exit hole. Adult emergence of *M. saltuarius* from May to June results in avoidance of the deleterious effects of high temperatures during post-diapause development in dead pine trees.

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DNA Fingerprints in the Honey Bee, *Apis mellifera*, Using an Ant-Derived DNA Probe¹

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A queen of the honey bee, *Apis mellifera*, mates with more than one male (ROBERTS, 1944; ALBER et al., 1955). If spermatozoa inseminated by multiple males fertilize eggs randomly, a colony should contain workers from many patrilines. In this case, the average relatedness within a colony decreases from 3/4

(single father) to approximately 1/4 (many fathers) because of haplo-diploid sex determination. It is important to determine whether workers discriminate between full and half sisters and cooperate more frequently with full sisters, and to understand the mechanism of the evolution and maintenance of altruism in social insects. To analyze genetic relationships in insects, some genetic markers are necessary. In honey bees, allozyme variation and polymorphism in body color have been used as markers (LAIDLAW and PAGE, 1984). Those markers, however, have a limitation in their ability to discriminate genetic relationships among nest mates.

Recently, JEFFREYS et al. (1985) discovered highly polymorphic minisatellite DNA in humans, and the determination of kinship has been made possible using this as a probe for southern hybridization (DNA fingerprinting). In the honey bee, DNA fingerprints have been obtained using synthetic oligonucleotide (GATA)₄ (MORITZ et al., 1991; HABERL and MORITZ, 1994) and phage M13 (BLANCHETOT, 1991) as probes, and FONDRK et al. (1993) analyzed polymorphism of

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randomly amplified DNA (RAPD). In the present study, we performed DNA fingerprint analysis using an ant-derived DNA fragment (pMY7) as a probe to determine the genetic relationship among the members of a colony.

MATERIALS AND METHODS

Sample collection. Colonies of the honey bee, *Apis mellifera*, were transferred from Kumagaya Apiary, Saitama Pref., and reared at the campus of Tokyo University of Agriculture and Technology, Tokyo, from 1992 to 1993. From colony A, one queen and 10 workers, from colony B, one queen, 11 workers and two drones and from colony C, one queen and 19 drones were collected and frozen at -70°C until DNA extraction. Additional queens, workers and drones were also collected from other colonies and stored for DNA fingerprinting of unrelated individuals in 1993.

DNA extraction. Each honey bee head was minced in a 1.5 ml tube in 500 μl of 5% citric acid. After mixing the contents, the tube was centrifuged at 5,000 r.p.m. for 5 min, and the supernatant was decanted; then 600 μl of solution A (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS) and 100 μl of solution B (100 mM Tris-HCl, 12.5 mM EDTA, 150 mM NaCl, 1% SDS, 1 $\mu\text{g}/\mu\text{l}$ Proteinase K) were added. The sample was incubated with gentle mixing at 56°C for 30 min and at 37°C over night. After an equal volume (700 μl) of phenol was added, the tube was inverted for 10 min, and centrifuged at 15,000 r.p.m. for 10 min. The supernatant was transferred to a new tube, and was extracted with an equal volume of phenol/chloroform, followed by additional extraction with an equal volume of chloroform. DNA was precipitated in an equal volume of isopropyl alcohol for 15 min at room temperature. The tube was centrifuged at 15,000 r.p.m. for 15 min, the supernatant decanted, and the precipitate washed twice with 500 μl of 70% ethanol. The DNA sample was desiccated and dissolved in 100 μl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Then 1 μl of RNase A (9 $\mu\text{g}/\mu\text{l}$) was added and the tube was incubated at 37°C overnight. To remove RNase, 300 μl of TE was added to the sample and was extracted with 400 μl of chloroform. The tube was inverted for 10 min, and centrifuged at 15,000 r.p.m. for 5 min. DNA was precipitated with 40 μl of 3.0 M sodium acetate and 1 ml of 100% ethanol for 20 min at -70°C , the supernatant decanted, and the precipitate washed twice with 500 μl of 70% ethanol. The DNA sample was

desiccated and dissolved in 100 μl of TE.

Restriction endonuclease digestion. Approximately 2 μg of DNA was extracted from each honey bee head. The DNA samples were digested with 12 units of *Hae*III at 37°C overnight, then extracted with phenol/chloroform, followed by chloroform extraction. Sample DNA was recovered by ethanol precipitation, using 1/10 volume of 3.0 M sodium acetate.

Southern blotting and hybridization conditions. Electrophoresis was carried out in a 1% agarose gel in circulating $1 \times \text{TAE}$ buffer (0.04 M Tris-acetate, 0.001 M EDTA (pH 8.0)) at 55 V for 18 h. The DNA was denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized in 0.5 M Tris-HCl (pH 6.7), 1.5 M NaCl, and transferred to a nylon membrane (Nytran, Schleicher & Schuell) by capillary blotting in $10 \times \text{SSC}$ buffer overnight. The membrane was UV crosslinked for 2 min and dried for 3 h at 70°C . Probe pMY7 was cloned from the genomic DNA of the ant *Manica yessensis* (MASUKO, unpublished). The probe was inserted in the plasmid (puc 118) and amplified by cultivating *E. coli* containing the plasmid. The probe was then labeled with ^{32}P -dCTP by random oligonucleotide priming. The length of the probe is 1.7 kb and it has many repeats of T (maximum number of T is 13) in various portions of its sequence. The membrane was prehybridized in 0.263 M Na_2HPO_4 , 1 mM EDTA, 7% SDS, 1% BSA, 1/100 volume of NONIDET P-40 (Sigma) at 65°C for 3 h (WESTNEAT et al., 1988), and hybridized with the probe (40 ng per reaction) in the same solution at 65°C for 12 h. The membrane was washed 4 times for 15 min in $2 \times \text{SSC}$, 0.1% SDS at 37°C , and was then exposed to an X-ray film (Fuji RX) for 5 to 7 days with an intensifying screen at -70°C .

Statistical analysis. We analyzed the scoreable DNA bands ranging from 2.0 to 23.0 kb. Band-sharing probabilities (KUHNLEIN et al., 1990) among individual focal categories were arcsine transformed to normalize the distribution. Then, the difference of the means was tested by a Student *t*-test. Average relatedness was estimated on the basis of band-sharing probability (REEVE et al., 1992).

RESULTS

Figure 1 shows the DNA fingerprints of a queen and 9 workers of colony A and a worker of an unrelated colony. The polymorphic bands of the queen's fingerprint were segregated among the workers of her colony. Apparently allelic bands were discernible, as indicated by paired arrows on the left side of the

Fig. 1. DNA fingerprints of a queen (Q) and 9 workers (lanes 1 to 9) from colony A and a worker (U) of another colony. M: size markers. Allelic bands are indicated by horizontal arrows. Paternal bands are indicated by downward or upward arrows.

queen's fingerprint. Paternal bands that were not discernible in the queen's fingerprint were discriminated in workers' fingerprints. Common bands which must have been derived from the same father were clearly distinguishable in workers #1, 3 and 5 (indicated by downward arrows) and in workers #2 and 7 (indicated by upward arrows). Fingerprints of colony B showed similar results.

Figure 2 shows the DNA fingerprints of a queen and 8 drones of colony C. All bands found in the drones' fingerprints were discernible in the queen's fingerprint. Apparently allelic bands were also discernible in those fingerprints (indicated by arrows). The mean number ($\pm$ S.D.) of fingerprint bands was $13.7 (\pm 3.7)$ in females ($n = 32$) and $6.2 (\pm 1.0)$ in males ($n = 21$). The mean number of bands found in the males' fingerprints was significantly lower (t -test, $t = 8.942$, $df = 51$, $p < 0.001$) (about half).

Band sharing probabilities among non-nestmate

Fig. 2. DNA fingerprints of a queen (Q) and 8 drones (lanes 1 to 8). M: size markers. Allelic bands are indicated by horizontal arrows.

Table 1. Band-sharing probabilities between non-nestmate (unrelated) honey bees and those between related honey bees (Q: queen, W: worker)

	Mean	S.D.	N
Non-nestmates	0.305	0.079	21
Colony A Q-W	0.700	0.064	9
W-W	0.579	0.100	36
Colony B Q-W	0.653	0.146	11
W-W	0.505	0.176	55

females and those among nestmate females are shown in Table 1. Mean band sharing probability among non-nestmates was significantly lower than that among nestmates (t -test: non-nestmates vs. between queens and their workers, $t = 11.931$, $df = 39$, $p < 0.001$; non-nestmates vs. among nestmate workers, $t = 6.581$, $df = 110$, $p < 0.001$). Within the same

colonies, average band-sharing probabilities between queens and workers were significantly higher than those among workers (*t*-test: colony A, $t = 3.433$, $df = 43$, $p < 0.005$; colony B, $t = 2.599$, $df = 64$, $p < 0.05$). In colonies A and B, estimates of average relatedness ($\pm$ S.D.) among nestmate workers were $0.395 (\pm 0.144)$ ($n = 36$) and $0.288 (\pm 0.254)$ ($n = 55$), respectively. The estimate of average relatedness among full-sisters judged by paternal band-sharing was significantly higher than that among half-sisters (0.605 ± 0.153 , $n = 11$ vs. 0.355 ± 0.146 , $n = 25$; $t = 4.833$, $df = 34$, $p < 0.001$).

DISCUSSION

DNA fingerprints using probe pMY7 showed highly polymorphic band patterns in *A. mellifera*. The bands in those fingerprints are apparently more polymorphic and so are distinctly separated as compared with those using the probes previously reported (BLANCHETOT, 1991; MORITZ et al., 1991; HABERL and MORITZ, 1994). The RAPD method (FONDRK et al., 1993) has problems in its reproducibility and the occurrence of non-parental bands. Fingerprints using pMY7 had high reproducibility and did not have non parental bands: the bands of drone's fingerprints were always seen in a queen's fingerprints. By analyzing those fingerprints, we could discriminate between full and half sisters as well as unrelated and related honey bees. For example, we could easily discern two groups of full sisters in the colony A (Fig. 1).

It is worthwhile to note that our results agree with the expectation from haplo-diploid sex determination in which males develop from unfertilized eggs parthenogenetically. All bands found in the drones' fingerprints were attributable to their mother queen (Fig. 2), and the number of fingerprint bands in males was about half that in females.

The fact that average band sharing probabilities among workers were significantly lower than those between queens and workers (Table 1) indicates functional polyandry. Intracolony estimates of average relatedness were 0.395 and 0.288 in colonies A and B, comparable to those estimated by LAIDLAW and PAGE (1984), MORITZ (1986) and HABERL and MORITZ (1994). These low values suggest that spermatozoa inseminated by many drones are mixed in a queen's

spermatheca and are used randomly in the fertilization of new eggs (LAIDLAW and PAGE, 1984; SASAKI et al., 1995).

Our study revealed that the ant-derived DNA probe, pMY7, was also very useful for DNA fingerprinting in the honey bee, *Apis mellifera*. Hybridization with the probe pMY7 produced a highly variable banding pattern in various ant species, e.g., *Formica japonica* (MURAKAMI et al., unpublished), *Camponotus nawai* and *C. yamaokai* (SATO et al., unpublished), and the parasitic Ichneumonid wasp *Venturia canescens* (OHYASHI and SATO, unpublished). Thus, we expect that the pMY7 probe will be useful for performing DNA fingerprinting in a wide variety of hymenopteran insects.

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