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# Intraspecific variation of cuticular hydrocarbon composition in *Formica japonica* Motschoulsky (Hymenoptera: Formicidae)

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**ABSTRACT**—Cuticular hydrocarbons and morphological features were compared among 80 *Formica japonica* colonies collected in Japan. Although a few morphological differences were found in workers among the colonies, four different types of cuticular hydrocarbon composition were observed. This was supported by a principal component analysis. We further compared the cuticular hydrocarbons among a total of approximately 400 *F. japonica* colonies, and categorized the hydrocarbon components into four types based on the result of discriminant analyses for the first 80 colonies. Type 1 was observed in colonies mainly collected in southern Honshu, Shikoku, and Kyushu. Types 2, 3, and 4 were from colonies with primary collections in Southern Honshu, central and Pacific coast northern Honshu, and the Sea of Japan coasts of northern Honshu and Hokkaido, respectively. The occurrence of four distinct types of CHC composition suggests that the colonies that produce them are separate species.

**Key words:** cuticular hydrocarbon, chemotaxonomy, *Formica japonica*, GC-MS

## INTRODUCTION

*Formica* (*Serviformica*) *japonica* Motschoulsky, 1866, is distributed in the Russian Far East, Mongolia, China, Korea, Japan, and the mountains of Taiwan. It is one of the most common ant species in Japan. It builds both monogynous and polygynous colonies that usually consist of a queen(s) and hundreds to thousands of workers and broods. The workers are usually solitary foragers. Their nestmate recognition is based on chemical signals that are believed to be cuticular hydrocarbon (CHC) blends (Yamaoka, 1990). The blends are shared by the colony members but differ among colonies. In the monogynous colony, the queen unifies the CHC blends of individual workers but the mechanism is still unknown (Yamaoka and Kubo, 1990).

In a citrus garden in Kainan City, Wakayama Prefecture, Japan, we sometimes observed that hundreds of *F. japonica* workers covered the ground and foraged simultaneously. The foraging style appeared different from that of the *F. japonica* workers we usually observed in Kyoto. The nest of the Wakayama garden colony was wider but more shallow, and contained dozens of inseminated queens. Mor-

phological features of the workers were almost identical to those of *F. japonica* in Kyoto, but the cuticular hydrocarbon components apparently differed. We therefore suspected that we were observing more than one species.

In general, a chemotaxonomical approach including cuticular hydrocarbon comparison is valuable for identification of sibling or cryptic species (Howard, 1993). The usefulness of the cuticular hydrocarbon comparison as the taxonomical criterion was also confirmed in a recent chemotaxonomical study on the termite genus *Reticulitermes* in eastern Asia (Takematsu and Yamaoka, 1999). In this study we compared both CHC components and morphological features among *F. japonica* colonies collected throughout all of the prefecture of Japan (except for Okinawa, where this species is absent), and discuss the possibility that *F. japonica* includes several sibling species.

## MATERIALS AND METHODS

From 1992 to 2001, we collected *Formica japonica* workers from all Japanese prefectures except Okinawa. At least 10 workers from each of 3 colonies, but many more if possible, were collected to check intracolony and intercolony variation of both morphological features and CHC in each district. We also collected *F. hayashi* workers because it is currently in the same subgenus and is sympatric in distribution with *F. japonica* (Terayama and Hashimoto,

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1996).

Individual live workers were anesthetized by cooling in a refrigerator and immersion in 500 µl of hexane for 5 min. The extract was concentrated and chromatographed on approximately 500 mg of silica gel (230–400 mesh, Merck), and packed in a disposable Pasteur pipette (7 mm dia.). The hydrocarbons were eluted with 3 ml of hexane, then analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

For identification of the position of the double bonds in unsaturated hydrocarbons, the separated hydrocarbons were further chromatographed on approximately 500 mg of silica gel, 230–400

mesh, and impregnated with 10% silver nitrate. Then they were successively eluted with 3 ml each of hexane, 1%, 2%, 5%, 10%, 15%, and 30% ether-in-hexane to separate unsaturated hydrocarbons from saturated ones. The unsaturated hydrocarbons were delivertized by dimethyl disulphide (DMDS) to estimate the position of unsaturated C-C bonds (Dunkelblum *et al.*, 1985)

GC analyses were performed on a Hewlett Packard HP6890 GC equipped with a flame ionization detector. An apolar capillary column (HP-1, Hewlett Packard, 15 m length, 0.25 mm dia., and 0.25 µm film thickness) was used for the analyses. Helium was used as a carrier gas, and the column head pressure was 60 kPa.

**Table 1.** Comparison of four CHC patterns in *Formica japonica*

| Peak No. | Hydrocarbon groups* <sup>1</sup> | ECL   | Suspected compounds               | Content** <sup>2</sup> |        |        |        |
|----------|----------------------------------|-------|-----------------------------------|------------------------|--------|--------|--------|
|          |                                  |       |                                   | Type 1                 | Type 2 | Type 3 | Type 4 |
| 1        | C23                              | 22.66 | 7-tricosene                       | *                      |        |        |        |
| 2        |                                  | 22.73 | 9-tricosene                       | *                      |        |        | *      |
| 3        |                                  | 23.00 | n-tricosane                       | *                      |        | *      | *      |
| 4        | MeC23                            | 23.40 | 11-methyltricosane                |                        |        |        | *      |
| 5        |                                  | 23.43 | 9-methyltricosane                 |                        |        |        | *      |
| 6        |                                  | 23.43 | 7-methyltricosane                 |                        |        |        | *      |
| 7        |                                  | 23.55 | 5-methyltricosane                 |                        |        | *      | *      |
| 8        | C24                              | 24.00 | n-tetracosane                     | *                      |        |        | *      |
| 9        | C25                              | 24.66 | 7-pentacosene                     | +                      |        |        |        |
| 10       |                                  | 24.73 | 9-pentacosene                     | +                      |        | *      | *      |
| 11       |                                  | 25.00 | n-pentacosane                     | +                      | *      | +      | *      |
| 12       | MeC25                            | 25.40 | 13-methylpentacosane              | +                      |        | *      | +      |
| 13       |                                  |       | 11-methylpentacosane              |                        |        |        |        |
| 14       |                                  | 25.43 | 9-methylpentacosane               |                        |        |        | +      |
| 15       |                                  | 25.54 | 5-methylpentacosane               |                        |        | *      | *      |
| 16       |                                  | 25.73 | 3-methylpentacosane               | *                      |        |        | +      |
| 17       | diMeC25                          | 25.68 | 9,x-dimethylpentacosane           |                        |        | *      | +      |
| 18       |                                  | 25.73 | dimethylpentacosane               | *                      |        |        |        |
| 19       |                                  | 25.82 | 5,X-dimethylpentacosane (X=12,13) |                        |        | *      | *      |
| 20       |                                  | 26.12 | 3,X-dimethylpentacosane (X=9,16)  |                        |        |        | *      |
| 21       | C26                              | 25.74 | 9-hexacosene                      |                        | *      |        |        |
| 22       |                                  | 26.00 | n-hexacosane                      | *                      | *      | *      | *      |
| 23       | MeC26                            | 26.39 | 13-methylhexacosane               | *                      |        |        |        |
| 24       |                                  |       | 12-methylhexacosane               | *                      |        |        |        |
| 25       |                                  |       | 10-methylhexacosane               |                        |        |        | *      |
| 26       | C27                              | 26.68 | 7-heptacosene                     | *                      |        | *      |        |
| 27       |                                  | 26.73 | 9-heptacosene                     | +                      | ++++   | +      | +      |
| 28       |                                  | 27.00 | n-heptacosane                     | +                      | +      | +      | +      |
| 29       | MeC27                            | 27.38 | 13-methylheptacosane              |                        | *      | +      | +      |
| 30       |                                  |       | 11-methylheptacosane              | ++                     |        |        |        |
| 31       |                                  | 27.41 | 9-methylheptacosane               |                        | *      | +      | +      |
| 32       |                                  | 27.52 | 5-methylheptacosane               |                        |        | +      | +      |
| 33       | diMeC27                          | 27.65 | 11,15-dimethylheptacosane         | +                      |        |        |        |
| 34       |                                  | 27.65 | 9,X-dimethylheptacosane           | +                      |        | +      | +      |
| 35       |                                  | 27.78 | 5,X-dimethylheptacosane           |                        |        | +      | +      |
| 36       | triMeC27                         | 28.11 | trimethylheptacosane              |                        |        |        | +      |
| 37       | C28                              | 27.75 | 9-octacosene                      |                        | *      |        |        |
| 38       |                                  | 28.00 | n-octacosane                      | *                      | *      | +      |        |
| 39       | meC28                            | 28.33 | 14-methyloctacosane               | *                      |        | *      |        |
| 40       |                                  | 28.35 | 12-methyloctacosane               |                        |        |        | +      |

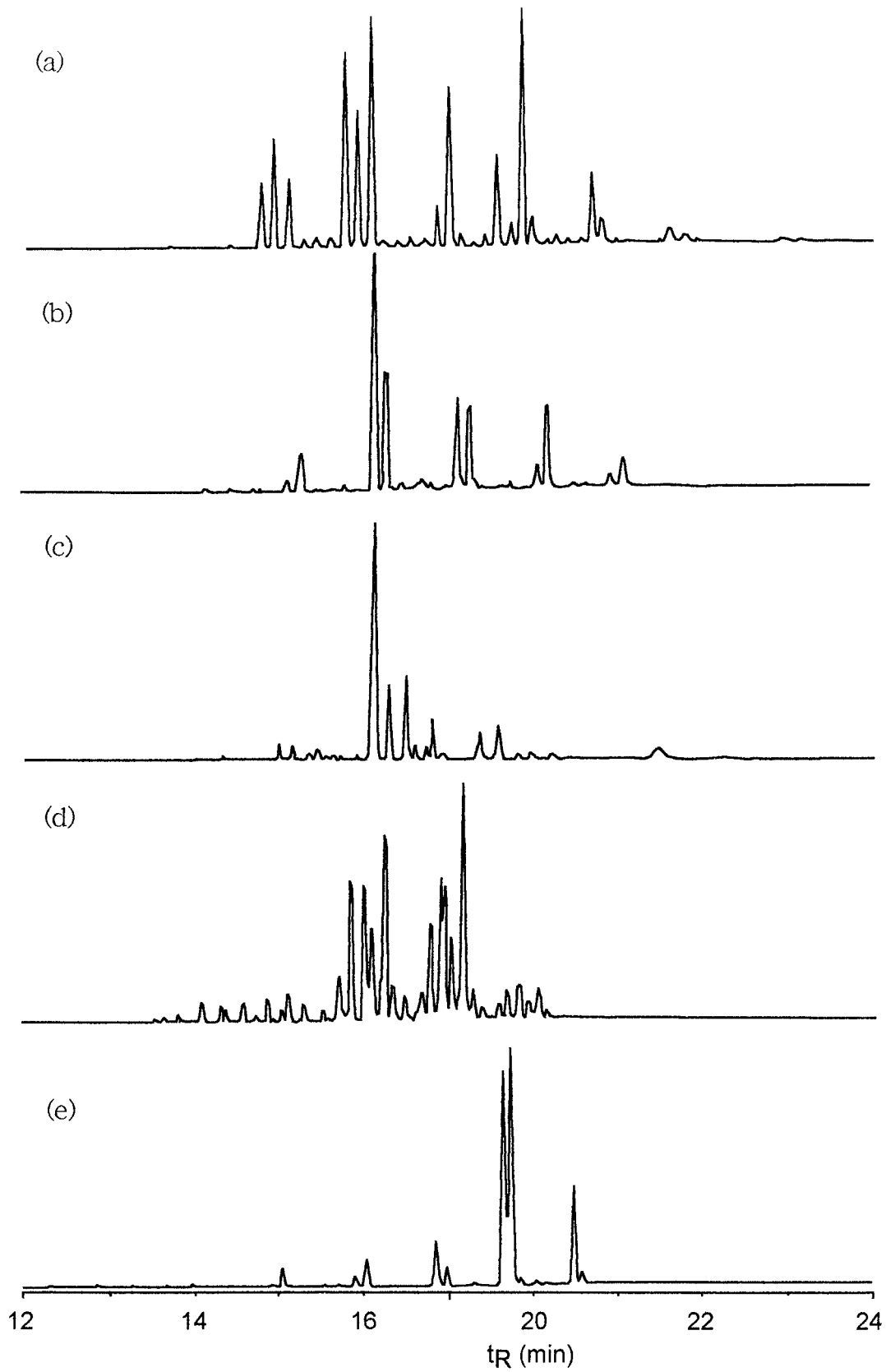
**Table 1.** (Continued)

| Peak No. | Hydrocarbon groups <sup>*1</sup> | ECL   | Suspected compounds          | Content <sup>*2</sup> |        |        |        |
|----------|----------------------------------|-------|------------------------------|-----------------------|--------|--------|--------|
|          |                                  |       |                              | Type 1                | Type 2 | Type 3 | Type 4 |
| 41       | diMeC28                          | 28.67 | 10,14-dimethyloctacosane     |                       |        |        | +      |
| 42       | C29                              | 28.63 | 7-nonacosene                 | *                     |        |        |        |
| 43       |                                  | 28.72 | 9-nonacosene                 | *                     | ++     | +      |        |
| 44       |                                  | 29.00 | n-nonacosane                 | +                     | +      | +      | +      |
| 45       | MeC29                            | 29.37 | 15-methylnonacosane          | +                     | *      | +      | +      |
| 46       |                                  | 29.37 | 13-methylnonacosane          | ++                    | *      | +      | +      |
| 47       |                                  | 29.37 | 11-methylnonacosane          | ++                    | *      | +      | +      |
| 48       |                                  | 29.41 | 9-methylnonacosane           |                       | *      | +      | +      |
| 49       | diMeC29                          | 29.62 | 11,X-dimethylnonacosane      | +                     |        |        |        |
| 50       |                                  | 29.68 | 9,X-dimethylnonacosane       |                       |        | +      | ++     |
| 51       |                                  | 29.76 | 5,X-dimethylnonacosane       |                       |        | +      | +      |
| 52       |                                  | 29.98 | dimethylnonacosane           | *                     |        |        |        |
| 53       | triMeC29                         | 30.03 | trimethylnonacosane          |                       |        |        | +      |
| 54       | C30                              | 29.74 | 9-triacontene                |                       | *      |        |        |
| 55       |                                  | 30.00 | n-triacontane                |                       | *      |        |        |
| 56       | MeC30                            | 30.36 | 14-methyltriacontane         | *                     |        |        | *      |
| 57       |                                  | 30.36 | 12-methyltriacontane         |                       |        |        | *      |
| 58       | diMeC30                          | 30.61 | 10,X-dimethyltriacontane     | *                     |        |        | +      |
| 59       | C31                              | 30.76 | 9-hentriacontene             |                       | *      |        |        |
| 60       |                                  | 31.00 | n-hentriacontane             | *                     | *      |        | *      |
| 61       | MeC31                            | 31.36 | 15-methylhentriacontane      |                       |        |        |        |
| 62       |                                  |       | 13-methylhentriacontane      |                       |        |        |        |
| 63       |                                  |       | 11-methylhentriacontane      | ++                    |        | +      | +      |
| 64       |                                  | 31.40 | 9-methylhentriacontane       |                       |        | +      | +      |
| 65       | diMeC31                          | 31.63 | 13,X-dimethylhentriacontane  | ++                    |        |        |        |
| 66       |                                  | 31.63 | 11,X-dimethylhentriacontane  | ++                    |        | +      |        |
| 67       |                                  | 31.66 | 9,X-dimethylhentriacontane   |                       |        |        | ++     |
| 68       |                                  | 31.88 | 5,X-dimethylhentriacontane   |                       |        | +      |        |
| 69       |                                  | 32.03 | 3,X-dimethylhentriacontane   |                       |        |        | +      |
| 70       | MeC32                            | 32.31 | methyl-dotriacontane         | *                     |        |        | *      |
| 71       | diMeC32                          | 32.61 | 10,X-dimethyl-dotriacontane  |                       |        |        | *      |
| 72       | C33                              | 32.75 | 9-tritriacontene             |                       | *      |        |        |
| 73       |                                  | 33.00 | n-tritriacontane             | *                     |        |        |        |
| 74       | MeC33                            | 33.33 | 17-methyltritriacontane      | +                     |        |        | +      |
| 75       |                                  | 33.33 | 15-methyltritriacontane      | +                     |        |        | +      |
| 76       |                                  | 33.34 | 13-methyltritriacontane      | +                     |        | *      | +      |
| 77       |                                  | 33.35 | 11-methyltritriacontane      | +                     |        | *      | +      |
| 78       |                                  | 33.37 | 9-methyltritriacontane       |                       |        |        | +      |
| 79       | diMeC33                          | 33.56 | 15,17-dimethyltritriacontane | +                     |        |        |        |
| 80       |                                  | 33.56 | 13,X-dimethyltritriacontane  | +                     |        | +      |        |
| 81       |                                  | 33.56 | 11,X-dimethyltritriacontane  | +                     |        | +      |        |
| 82       |                                  | 33.72 | dimethyltritriacontane       |                       |        |        | +      |
| 83       | MeC35                            | 35.39 | 17-methylpentatriacontane    | *                     |        |        |        |
| 84       |                                  | 35.39 | 15-methylpentatriacontane    | *                     |        |        |        |
| 85       |                                  | 35.40 | 13-methylpentatriacontane    | *                     |        |        |        |
| 86       | diMeC35                          | 35.71 | dimethylpentatriacontane     | +                     |        |        |        |
| 51       |                                  | 29.76 | 5,X-dimethylnonacosane       |                       |        | +      | +      |
| 52       |                                  | 29.98 | dimethylnonacosane           | *                     |        |        |        |
| 53       | triMeC29                         | 30.03 | trimethylnonacosane          |                       |        |        | +      |

<sup>\*1</sup> Carbon number in the longest carbon chain is noted after the large capital C. For methylalkanes, the number of methyl branches is shown as "(mono)Me," "diMe," and "triMe" before the capital "C".

<sup>\*2</sup> Contents by percentage: \* <1%, + <10%, ++ <20%, +++ <30%, and ++++ <40%

Type 1, 2, 3, and 4 workers were collected in Tsuno (Miyazaki Prefecture), Kyoto (Kyoto Prefecture), Nasu (Tochigi Prefecture), and Hakodate (Hokkaido Prefecture), respectively.



**Fig. 1.** Gas chromatogram of CHC blends of *Formica japonica* workers collected in Tsuno (Miyazaki Pref.) (a), Kyoto (Kyoto Pref.) (b), Nasu (Gunma Pref.) (c), Hakodate (Hokkaido Pref.) (d), and of *Formica hayashi* workers (e)

Injection was made directly onto the capillary column through the cool on-column injector at 53°C and the injector temperature was programmed at oven temperature plus 3°C thereafter. The temperature program of column oven temperature was 50°C for 5 min, 50°C to 310°C at 15°C/min, and then held at the final temperature for 5 min.

GC-MS analyses were achieved with an HP6890 gas chromatograph interfaced to a JEOL JMS SX-102A double focusing mass spectrometer at EI mode with 70 eV, and operated with an HP Model 715/64 computer. GC was operated in the same condition as above, but the column head pressure was 18 kPa.

Nei's distance (Ferguson, 1980) was calculated as an index of the similarity of CHC blends for individual workers from a total of 40 colonies. Nei's distance  $c$  was defined as  $c = |\mathbf{a}-\mathbf{b}|/(\mathbf{a}\cdot\mathbf{b})$  where  $\mathbf{a}$  and  $\mathbf{b}$  are vectors. Each vector presents the CHC blend of individual workers that is composed of 86 elements. The elements are the area size for selected FID peaks with amounts greater than 0.1% of the total amount of the CHC's. For a larger value of  $c$ , similarity is considered to increase, and *vice versa*.

A total of 53 hydrocarbon components with relative content that was more than 1% of the total amount in each colony were selected for multivariate analysis. The data were processed by cluster analysis (CA) and principal component analysis (PCA) and were submitted to discriminant analysis (DA) using Mahalanobis distance. All these multivariate analyses were performed with the "Black-Box package" for data analyses in Aoki, 2001.

## RESULTS

### Identification of CHC components

CHC compounds of *F. japonica* consisted of alkenes, *n*-alkanes and various methylalkanes with 23–37 carbons. At least four different types of CHC pattern were confirmed among *F. japonica* colonies (Fig. 1a–d, Table 1).

The CHC components of the workers collected in Tsuno (Miyazaki Prefecture) were with 23–37 carbons. Alkenes contained 7-alkenes and 9-alkenes, and methylalkanes consisted of mono-, di-, and trimethylalkanes (Fig. 1a) (hereafter, named Type 1). Those of the workers collected in Kyoto (Kyoto) were, however, with 23–33 carbons. The alkenes were all 9-alkenes, and methylalkanes were

minor components (Fig. 1b) (Type 2). The CHC components of the workers in Nasu (Tochigi) were with 23–35 carbons, of which methylalkanes were mono- and dimethylalkanes (Fig. 1c) (Type 3). Those of the workers in Hakodate (Hokkaido) were also with 23–35 carbons, but the methylalkanes contained trimethylalkanes in addition to mono- and dimethylalkanes (Fig. 1d) (Type 4).

In contrast, the CHC patterns of *F. hayashi* were all identical among the colonies collected in Hakodate (Hokkaido), Hachinohe (Aomori), Hanamaki (Iwate), Mito (Ibaraki), Suwa (Nagano), Kyoto (Kyoto), Nakamura (Kouchi), Fukuoka (Fukuoka), and Osumi (Kagoshima). The CHC consisted of alkadiene, alkene, and *n*-alkanes with 25–33 carbons (Fig. 1e).

### Intra- and intercolonial variation of the CHC blends

Resemblance of the CHC blends was evaluated as Nei's distance among 10 nestmate workers each from colonies of Miyazaki, Kyoto, Tochigi and Hokkaido Prefectures (all sites had different CHC types). Nei's distance was calculated between all the pairs of the 10 nestmate workers of each colony (45 pairs). Averages±standard error (s.e) were 0.983±0.003, 0.950±0.026, 0.984±0.010, and 0.977±0.015 in the colonies collected in Miyazaki, Kyoto, Tochigi, and Hokkaido, respectively (Table 2). In the same manner, Nei's distance was calculated between all the pairs of workers from different colonies (100 pairs between 2 colonies). The average distance was always smaller between the colonies with different CHC types than between the colonies with the same CHC types, but the latter was smaller than that among nestmate workers (*t*-test,  $P < 0.001$ , Table 2).

Cluster analysis (CA) (Ward's technique) was conducted on CHC data obtained from a total of 80 *F. japonica* colonies that were pairs of sympatric colonies collected from 40 different localities (Fig. 2). It suggests the existence of four principal clusters (Type 1, 2, 3, and 4) within the 80 *F. japonica* colonies. Colonies of Type 1 were collected in

**Table 2.** Resemblance of the cuticular hydrocarbon components within and among colonies with different CHC types

|        | Nei's distance (average±standard error) |                                  |                                  |                                  |
|--------|---|----------------------------------|----------------------------------|----------------------------------|
|        | Type 1                                  | Type 2                           | Type 3                           | Type 4                           |
| Type 1 | 0.983±0.003*<br>(0.917±0.015)***        | 0.444±0.189**                    | 0.647±0.103**                    | 0.556±0.068**                    |
| Type 2 |   | 0.950±0.026*<br>(0.909±0.026)*** | 0.510±0.219**                    | 0.369±0.191**                    |
| Type 3 |   |                                  | 0.984±0.010*<br>(0.928±0.014)*** | 0.666±0.096**                    |
| Type 4 |   |                                  |                                  | 0.977±0.015*<br>(0.936±0.024)*** |

\* Nei's distance was calculated between all pairs of 10 nestmate workers in each colony, and the distances of a total of 45 pairs were averaged.

\*\* Nei's distance was calculated between all pairs of 10 workers from different colonies, and the distances of a total of 100 pairs between two colonies were averaged.

\*\*\* Nei's distance was calculated between all pairs of five workers each from five different sympatric colonies, and the distances of a total of 125 pairs were averaged.

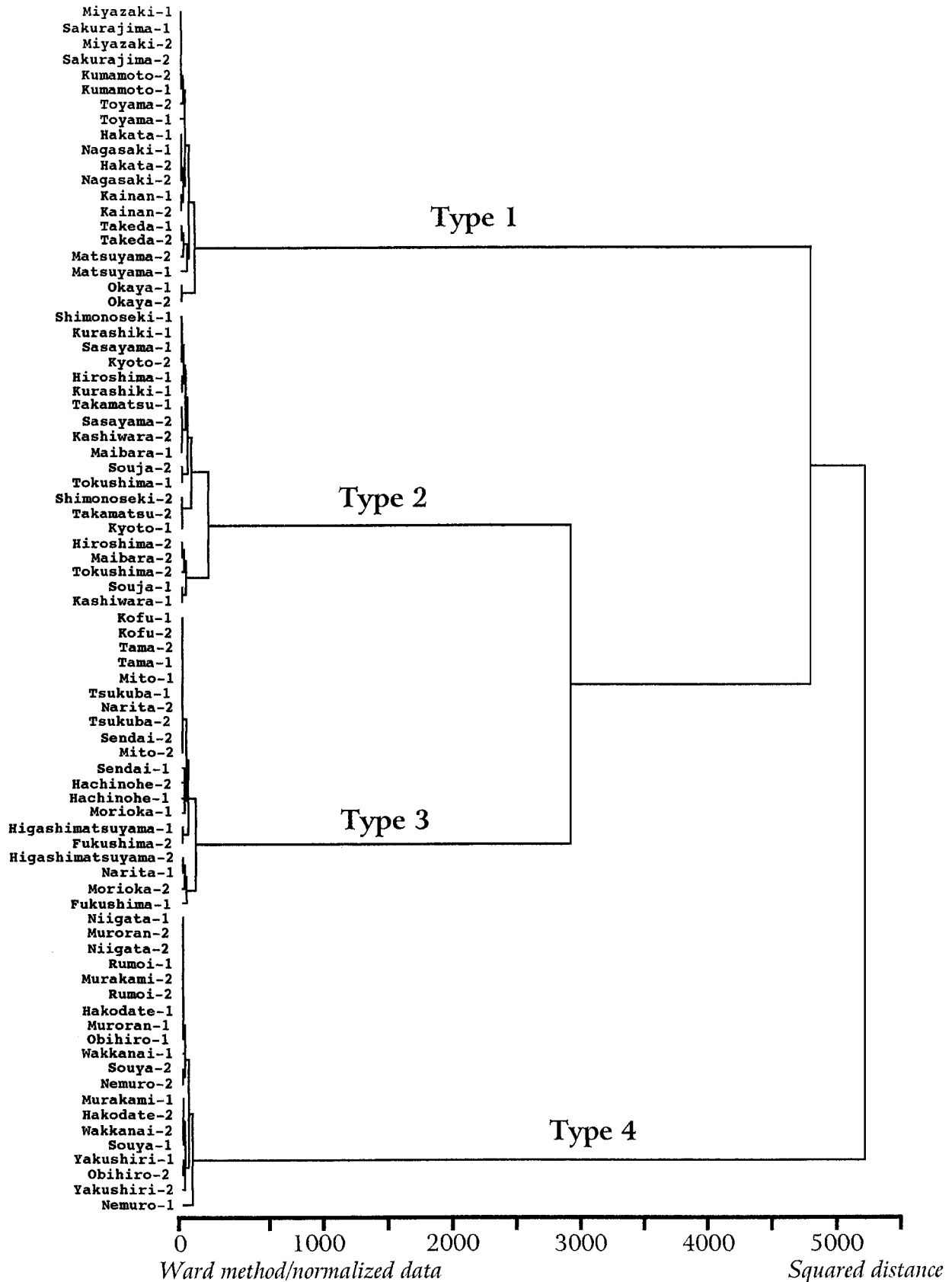
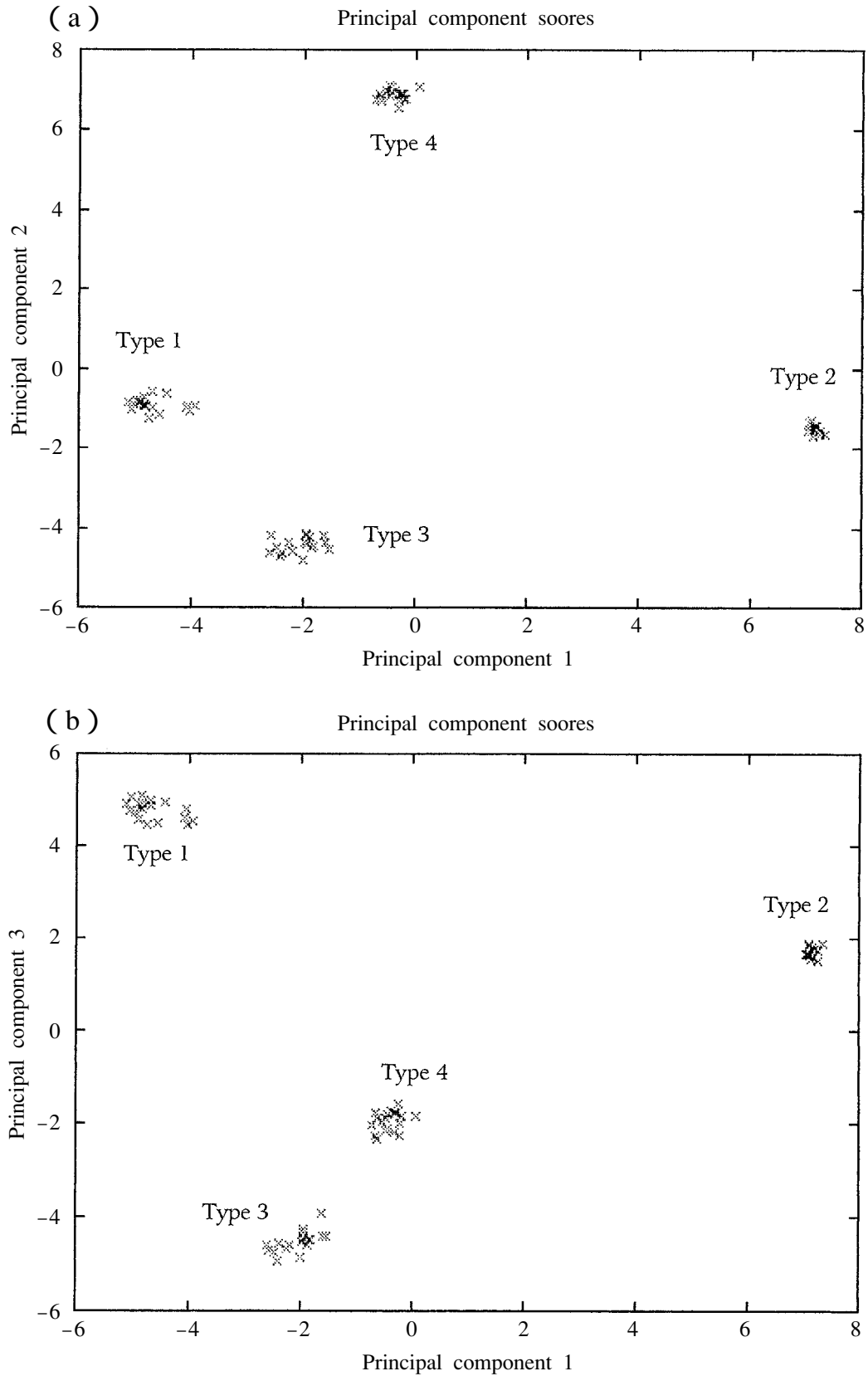


Fig. 2. Dendrogram from the cluster analyses of the original CHC blends



**Fig. 3.** Scatterplot of the 80 colonies for the 1st-2nd (a) and the 1st-3rd (b) principal components extracted in PCA



Miyazaki (Miyazaki), Sakurajima (Kagoshima), Nagasaki (Nagasaki), Kumamoto (Kumamoto), Matsuyama (Ehime), Hakata (Fukuoka), Takeda (Oita), Toyama (Toyama), Okaya (Nagano), and Kainan (Wakayama); those of Type 2 in Shimonoseki (Yamaguchi), Hiroshima (Hiroshima), Kurashiki (Okayama), Souja (Okayama), Takamatsu (Kagawa), Tokushima (Tokushima), Sasayama (Hyogo), Kyoto (Kyoto), Kashiwara (Nara), and Maibara (Shiga); those of Type 3 in Kofu (Yamanashi), Higashimatsuyama (Saitama), Tama (Tokyo), Narita (Chiba), Tsukuba (Ibaraki), Mito (Ibaraki), Fukushima (Fukushima), Sendai (Miyagi), Hachinohe (Aomori), and Morioka (Iwate); and those of Type 4 in Niigata, Murakami (Niigata), Hakodate, Muroran, Rumoi, Wakkanai, Souya, Yakishiri, Nemuro, and Obihiro (Hokkaido).

Principal component analysis (PCA) was also conducted on CHC data obtained from the 80 colonies. The

first, second, and third principal components accounted for 36%, 34%, and 24% of the total cumulative variance, respectively. Analysis of these three principal components shows that the colonies can be classified into four groups (Fig. 3). A plot of the first and second principal components, which accounted for 70% of the total cumulative variance, shows that both Type 2 and Type 4 colonies are well separated from both Type 1 and Type 3. In contrast, in a plot of the first and third principal components, Type 1 and Type 2 colonies are well separated from Type 3 and 4 colonies, whereas that of the second and third principal components of Type 3 and Type 4 colonies are well separated from Type 1 and Type 2.

As a second step, stepwise discriminant analysis (DA) using Mahalanobis distance was conducted on the CHC data from 80 colonies to determine which variables separate the four groups. The calibration consisted of the group mean

**Table 3.** Regression coefficient and partial F value for each hydrocarbon component as a significant variable for discrimination of four types by Mahalanobis distance

| Hydrocarbon components | Regression coefficient |        |        |        | Partial F value | P value |
|------------------------|------------------------|--------|--------|--------|-----------------|---------|
|                        | Type 1                 | Type 2 | Type 3 | Type 4 |                 |         |
| X002                   | -10561                 | 291    | -8559  | -5037  | 65.017          | 0.008   |
| X004                   | -169                   | -380   | -4018  | -1631  | 46.636          | 0.058   |
| X006                   | -5833                  | 448    | -946   | -7174  | 87.013          | 0.001   |
| X008                   | -13418                 | 766    | -10787 | -11162 | 200.4           | 0.000   |
| X009                   | 14151                  | -2114  | -855   | -28938 | 251.27          | 0.000   |
| X010                   | 2158                   | -1140  | 1779   | -3977  | 73.756          | 0.003   |
| X012                   | -5330                  | -214   | -7136  | 813    | 75.973          | 0.003   |
| X016                   | -930                   | 511    | 2143   | 22786  | 93.418          | 0.001   |
| X017                   | -14863                 | 303    | -12590 | -26595 | 120.09          | 0.000   |
| X018                   | 757                    | -502   | -4599  | -8582  | 164.79          | 0.000   |
| X019                   | -9140                  | -232   | -11738 | -14124 | 153.76          | 0.000   |
| X024                   | -1535                  | -505   | -2112  | 3081   | 33.452          | 0.258   |
| X028                   | -7272                  | 84     | -706   | -6477  | 127.46          | 0.000   |
| X029                   | 21424                  | -2282  | 9307   | -3075  | 311.03          | 0.000   |
| X031                   | 34660                  | -2549  | 20959  | -944   | 439.13          | 0.000   |
| X032                   | 4290                   | -397   | -10870 | 1050   | 101.76          | 0.000   |
| X037                   | -40701                 | 3283   | -29529 | 3601   | 1198.3          | 0.000   |
| X039                   | -38351                 | 2337   | -27825 | -693   | 1332.4          | 0.000   |
| X040                   | -29623                 | 537    | -31106 | -16140 | 519.75          | 0.000   |
| X044                   | -32054                 | 2074   | -30192 | 1717   | 685.08          | 0.000   |
| X045                   | -11397                 | -7     | 23280  | -61469 | 811.82          | 0.000   |
| X046                   | -22195                 | -871   | 2184   | -53021 | 1422            | 0.000   |
| X048                   | 13658                  | -927   | 7655   | 12391  | 118.17          | 0.000   |
| X049                   | -3866                  | -1376  | 2426   | -30714 | 323.78          | 0.000   |
| constant               | 253770                 | 4981.9 | 188460 | 384320 |                 |         |

Mahalanobis distances were 165 between Types 1 and 2, 118 between Types 1 and 3, 185 between Types 1 and 4, 138 between Types 2 and 3, 192 between Types 2 and 4, and 186 between Types 3 and 4.

matrix and the inversed pooled correlation matrix. Mahalanobis distances between the various groups were calculated for the data sets from which the calibrations were generated. These distances are displayed in Table 3. As a result of DA, a total of 24 hydrocarbon components were chosen as significant discriminators to predict the groups. Regression coefficients and partial F values are shown in Table 3.

**Distribution of colonies with four types of CHC's in Japan**

We classified CHC patterns of approximately 400 *F. japonica* colonies that were collected in Japan into four groups based on DA. Fig. 4 shows the distribution of the colonies with the four different types of CHC. Type 1 colonies

(white circles) were mainly distributed in southern Honshu, Shikoku, and Kyushu. Type 2 colonies (black circles) were distributed in southern Honshu, whereas Type 3 colonies (blue circles) are found in central and coastal (Pacific) northern Honshu. Type 4 (red circles) distribution is on the Sea of Japan coast of northern Honshu and Hokkaido. In several prefectures, the four types were sympatric.

**Comparison of morphological features**

We examined workers of the four types to find differences in external morphology by measuring head length (HL), head width (HW), antennal scape length (SL), compound eye length, cephalic index (HW/HL x 100), and scape index (SL/HW x 100). There were no significant differences in these morphological features of the workers among the

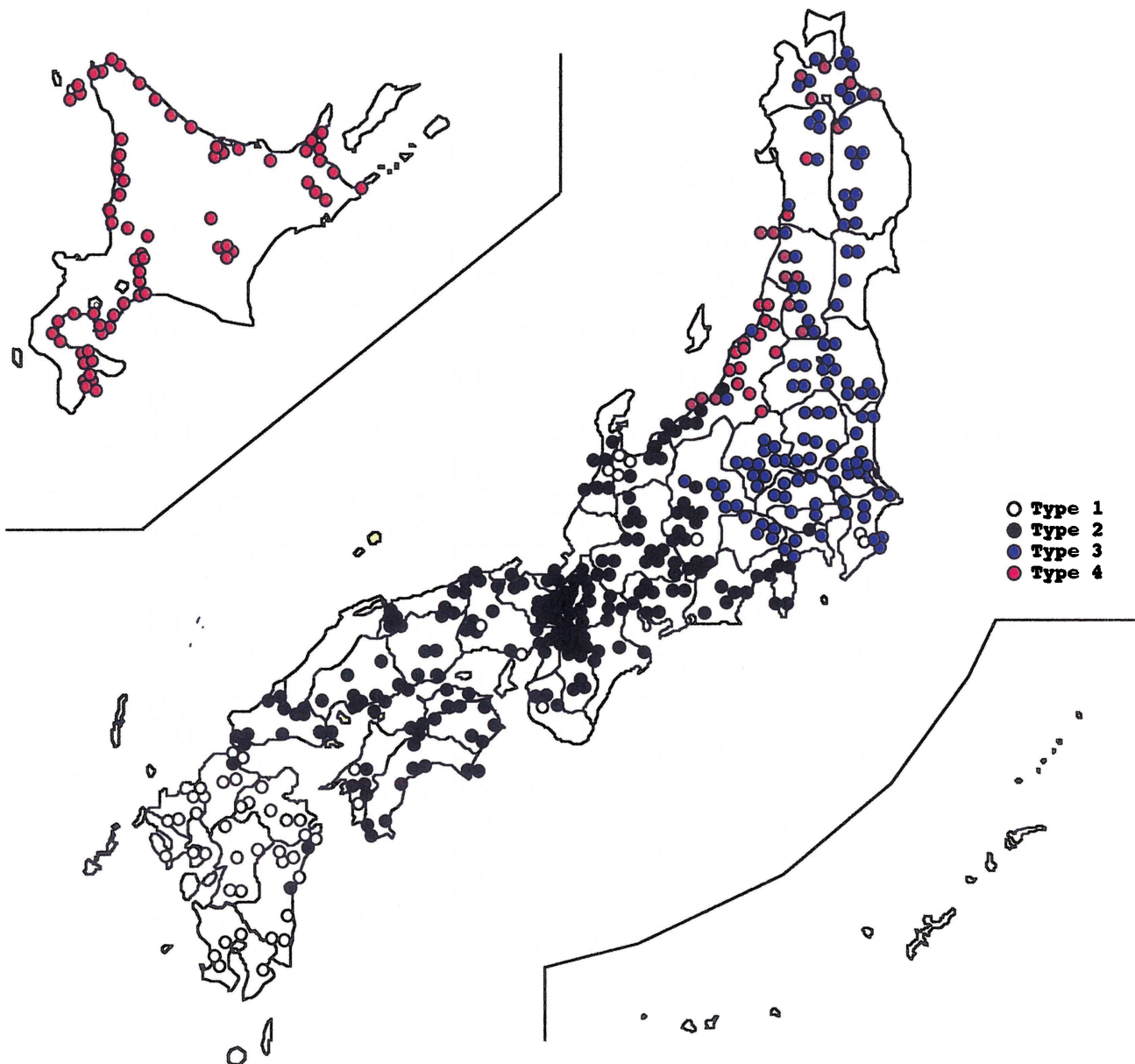


Fig. 4. Distribution of four types of CHC blends in Japan

types (Terayama, Akino and Yamaoka, in prep.).

The mounted voucher specimens of the four types of *F. japonica* are deposited in the National Institute of Agro-Environment Science, Tsukuba, and the Museum of Nature and Human Activities, Hyogo.

## DISCUSSION

Comparison of the CHC in *F. japonica* revealed that (1) nestmate workers shared almost identical blends that contained all the CHC components (Table 2), and (2) the CHC components were common but the blend ratios differed even among sympatric colonies. These results are consistent with previous studies on the CHC in ants (Howard 1993; Vander Meer and Morel 1998; Yamaoka 1990), *i.e.*, the ant CHC compositions are species specific and their blend ratios are colony specific. In this study, however, we found (3) four distinct types in CHC composition were present in *F. japonica* specimens collected throughout Japan (Types 1, 2, 3, and 4 in Table 1).

Cluster analyses on the CHC blends of 80 colonies suggested the existence of four principal groups (Fig. 2). PCA reduced the 53 variables to 3 principal components that represented 94% of the total variance. This also allowed us to establish four groups. The 1st, 2nd, and 3rd principal components characterized Type 2, Type 4, and Type 1, respectively. Subsequently, stepwise discrimination analyses using Mahalanobis distance determined 24 CHC components for discrimination of the four groups (Table 3). Classification of *F. japonica* colonies based on DA indicated that Type 1 was observed in colonies mainly distributed in southern Honshu, Shikoku, and Kyushu, Type 2 in southern Honshu, Type 3 in central and Pacific-coastal northern Honshu, and Type 4 at the Sea of Japan coast of northern Honshu and Hokkaido (Fig. 4). In *F. japonica* colonies, CHC blends are paralleled by the colonies, separate distributions. It appears that colonies of these four CHC types correspond to geographical populations, and that the four populations may be sibling species. As shown in Fig. 4, colonies with different CHC types are distributed sympatrically in several areas, including Nagano, Niigata, Toyama, and Yamaguchi Prefectures. We have not yet found hybrid types of CHC blends among the CHC types. If the differences of CHC components are due to intraspecific variation, hybrid types of CHC should be found in the places where colonies with different CHC types nest sympatrically. In contrast, if the differences are due to interspecific variation, hybrid CHC types would be seldom found. Further detailed research, especially at boundaries between different CHC types, is necessary to see if such crossbreeding occurs between reproductives of different CHC types.

In ants, there are many cases where workers of different populations are hardly separated by means of ordinary anatomical traits, although reproductive isolation is strongly suggested (Wilson, 1988; Hölldobler and Wilson, 1990). These sibling species are often separated by the differences

in the morphological features specific to the reproductive, *i.e.*, queens and males, or by the differences in chromosome numbers, biology, and/or ethology (Crozier, 1977; Crosland *et al.*, 1988; Halliday, 1981; Ward, 1980a,b, 1983; Seifert, 1991). Therefore, the actual number of extant ant species should be much greater than the current number described, because of these sibling species. This is also true for *Formica* (Vepsäläinen and Pisarski, 1981; Douwes, 1981). Although *F. hayashi* was formerly placed in the same taxon with *F. japonica*, slight habitat differences were observed. With this as a start, their morphology was carefully compared, which resulted in the delineation of two species (Kondoh, pers. comm.; Terayama and Hashimoto, 1996).

Although few minor morphological differences were recognized in workers among the four groups, our discussion recognizing distinct groups in *F. japonica* may nevertheless be justified. Howard (1993) indicated that insect CHC compositions are generally different at species level, and that there is little intraspecific variation. This provides a new dimension of CHC compositions as phenotypes for discrimination and identification of insect species. Such chemotaxonomical approaches have been used with beetles (Golden *et al.*, 1992; Lockey, 1991), moths (Carlson and Milstrey, 1991; Lavine and Carlson, 1991), termites (Kaib *et al.*, 1991; Haverty *et al.*, 1988; Takematsu and Yamaoka, 1999) and parasitic wasps (Espelie *et al.*, 1990). Many of these studies sought a better means to classify and identify sibling or cryptic species, in which morphological differences were few found. In most cases, the CHC indeed appears to be a valuable character for identification, and also a cue for further detailed morphological comparison.

In *F. japonica*, we propose that the four groups are independent species. Geographical separation among the four different CHC types may be a result of allopatric or parapatric speciation. If so, it is uncertain how it developed. Even if the differences are due to intraspecific variation, however, the phenomenon is still interesting because there are no reports that show such clear variation in the CHC components within species. To test our hypothesis, further studies are necessary in the following context: 1) comparison of the genetic distance among the four groups using molecular techniques, 2) comparison of the morphological features specific to reproductives, and 3) observation of reproductive barriers between the four groups by ecological and ethological methods.

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