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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. Morphological 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 intracolonial and intercolonial 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*1	ECL	Suspected compounds	Content*2			
				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*1	ECL	Suspected compounds	Content*2			
				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-hetriacontane	*	*		*
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	methyldotriacontane	*			*
71	diMeC32	32.61	10,X-dimethyldotriacontane				*
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				+

*1 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".

*2 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.

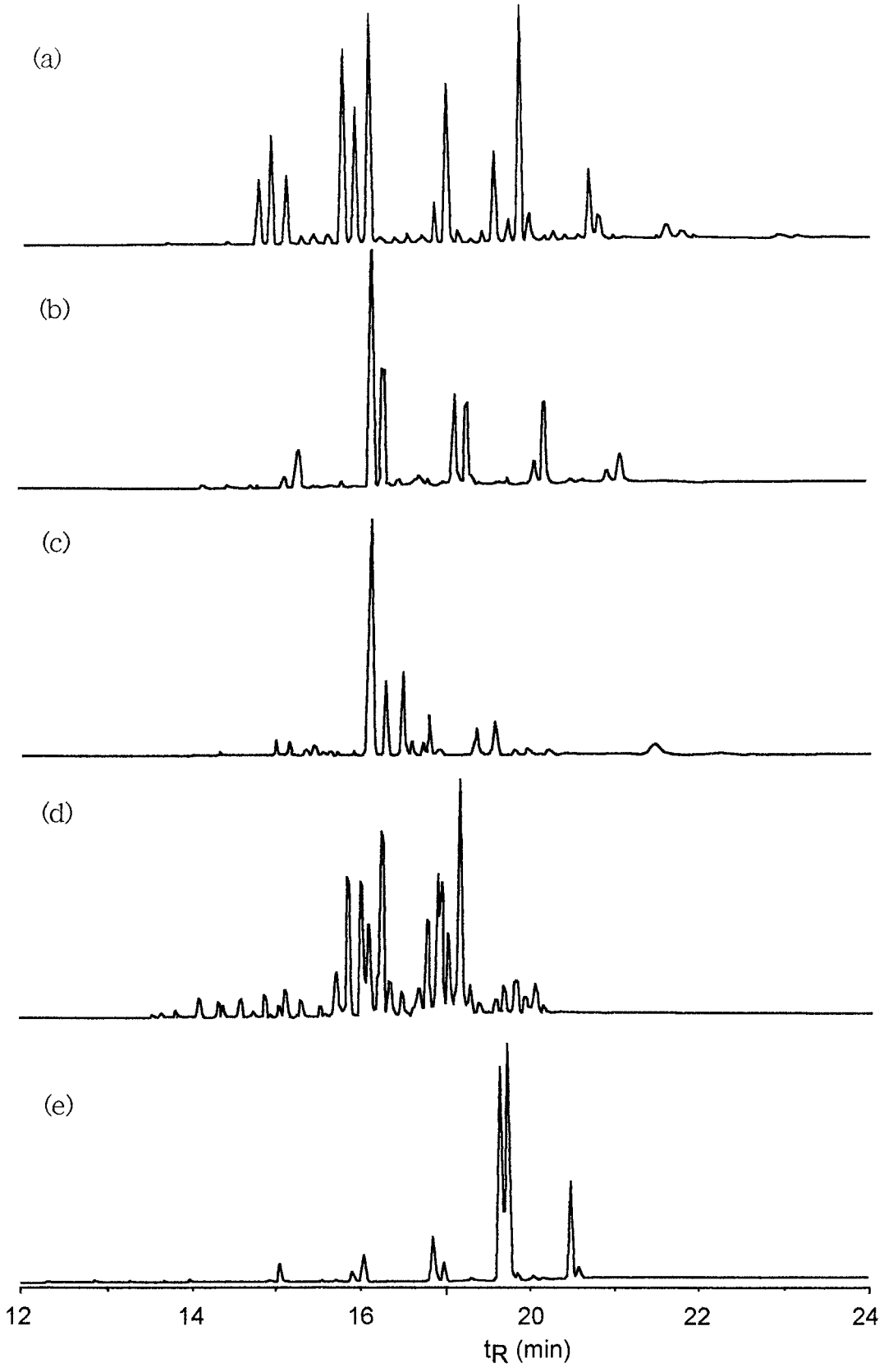

Fig. 1. Gas chromatogram of CHC blends of *Formica japonica* workers collected in Tsuno (Miyazaki Pref.) (a), Kyoto (Kyoto Pref.) (b), Nasu (Gunma Pref.) (c), Hakodate (Hokkaido Pref.) (d), and of *Formica hayashi* workers (e)

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 $c = |\mathbf{a}\cdot\mathbf{b}|/(\mathbf{a}\cdot\mathbf{b})$ where **a** and **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 multivariant 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* (0.917±0.015)***	0.444±0.189**	0.647±0.103**	0.556±0.068**
Type 2		0.950±0.026* (0.909±0.026)***	0.510±0.219**	0.369±0.191**
Type 3			0.984±0.010* (0.928±0.014)***	0.666±0.096**
Type 4				0.977±0.015* (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.

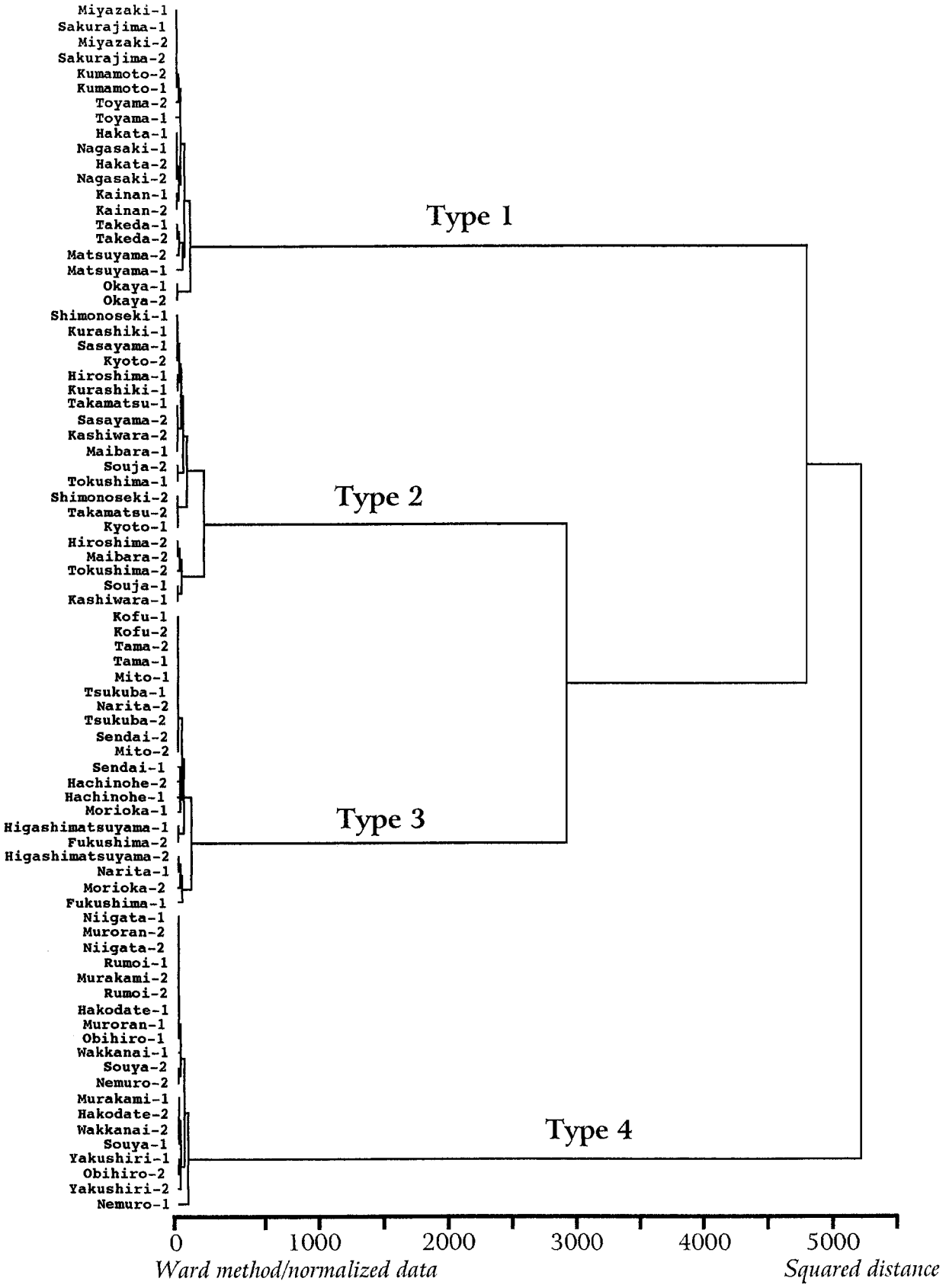

Fig. 2. Dendrogram from the cluster analyses of the original CHC blends

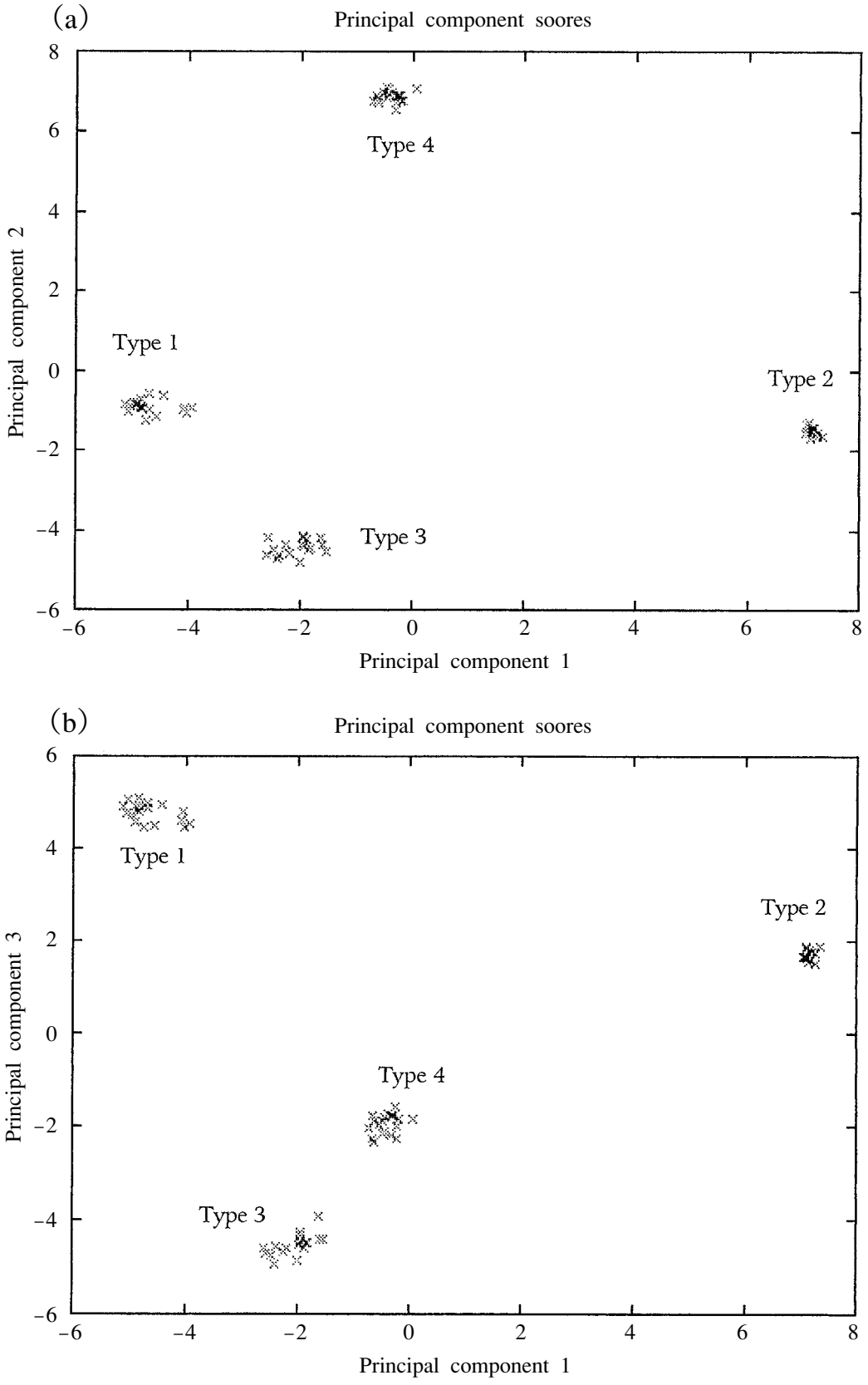

Fig. 3. Scatterplot of the 80 colonies for the 1st-2nd (a) and the 1st-3rd (b) principal components extracted in PCA

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

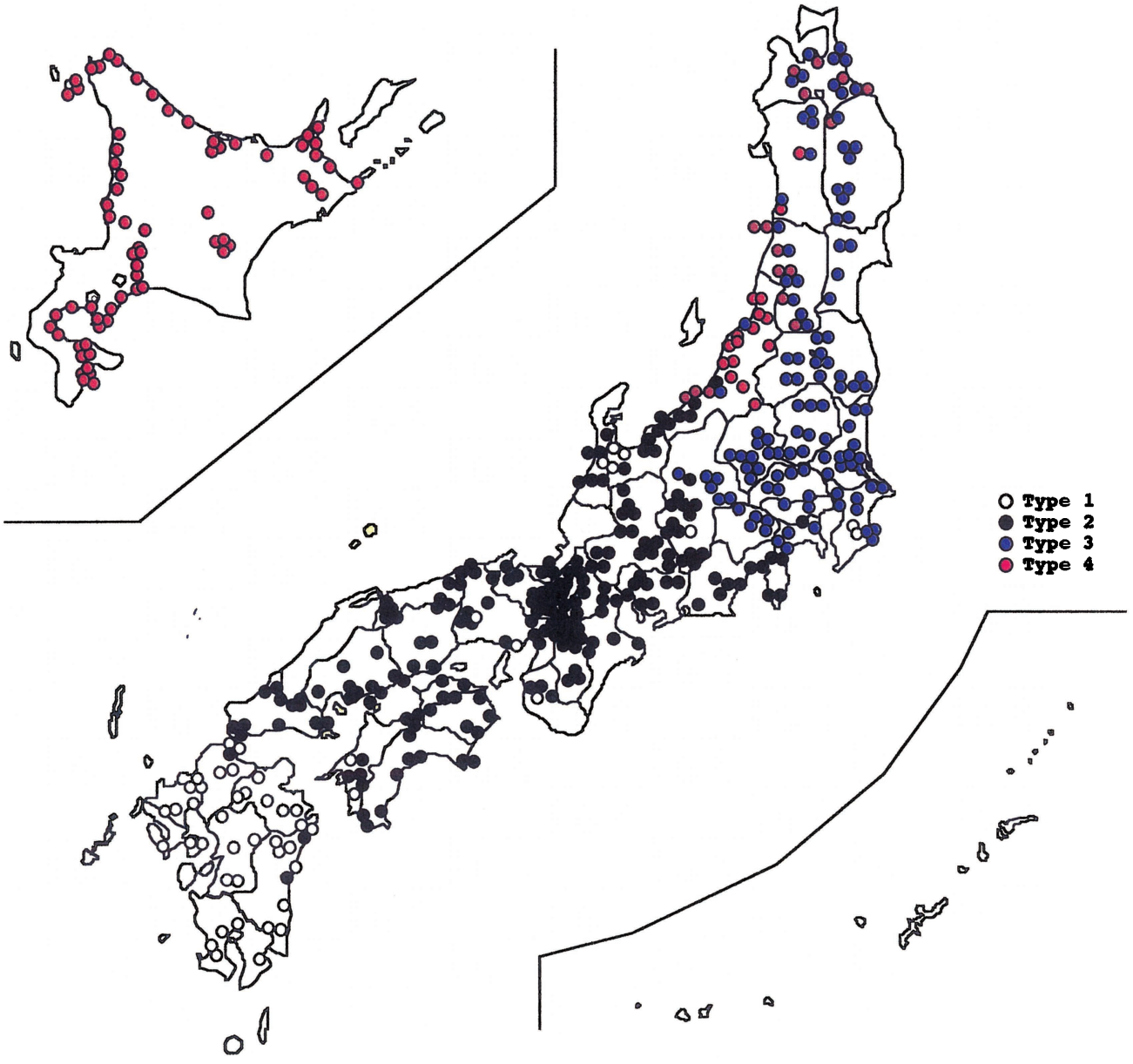

Fig. 4. Distribution of four types of CHC blends in Japan

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 pupulations, and that the four pupulations may be sibling species. As shown in Fig. 4, colonies with different CHC types are distributed sympatrically in several area, 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 f 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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