Identification of the Tandem Running Pheromone in *Diacamma* sp. from Japan (Hymenoptera, Formicidae)

by

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ABSTRACT

The Japanese queenless ponerine ant *Diacamma* sp. from Japan employs tandem running during nest relocation, in which a leader ant guides nestmate followers one at a time. We replicated this process by presenting one entire abdominal part of a leader, except for the petiole to followers. When the abdominal part had been rinsed with *n*-hexane, however, it attracted significantly fewer followers. This suggests that chemicals on the leader's abdominal part evoke tandem running. Dissection of abdominal major exocrine glands revealed that the Dufour's gland was the source of this chemical signal. The chemicals were eluted in the hydrocarbon fraction by silica-gel column chromatography, and the quantitatively major component was estimated as heptadecene ($C_{17:1}$) through gas chromatograph-mass spectrometer (GC-MS) analysis. The position of the double bond was estimated to be between the 8th and 9th carbons through analysis of the epoxidized compound. Only (*Z*)-isomers of 8-heptadecene evoked tandem running in the followers. We identified the tandem running pheromone of this ant species to be (*Z*)-8-heptadecene. (163)

Keywords: tandem running, nest emigration, chemical communication, Formicidae, (*Z*)-8-heptadecene.

INTRODUCTION

In many ant species, scout ants recruit their nestmates to newly discovered food sources and nesting sites, and also to battle sites at territorial borders. A variety of communication mechanisms comprise recruitment behavior of each

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ant species (Hölldobler & Wilson 1990). One behavior is known as tandem running, which is generally considered as one of the most primitive of these recruitment systems (Wilson 1971). In this system, each scout (leader) ant recruits only one nestmate at a time (Hölldobler & Traniello 1980a; Traniello & Hölldobler 1984). The tandem running seems to be triggered by a signal from the leader's gaster, which induces a following behavior when perceived by a nearby worker (follower). The signal is known as the tandem running pheromone. Followers are always strongly attracted to the dorsal parts of abdominal segments or hind legs of leaders. In some ant species, the sources of tandem running pheromone have been reported to exude from exocrine glands (such as the pygidial or poison gland; Möglich 1974; Hölldobler & Traniello 1980b). Nevertheless, chemical identification has not yet been completed for the tandem running pheromones in any ant species.

Tandem running recruitment is also observed in the Japanese queenless ponerine ants *Diacamma* sp. from Japan (Fukumoto & Abe 1983). They are very sensitive to environmental changes of nesting sites, and often move them in conjunction with tandem running recruitment. This sensitivity enabled us to develop a bioassay system to evaluate the tandem running activity in the laboratory and preliminary observation confirmed that a single fresh abdomen dissected from a leader evoked this behavior. The behavior was not evoked after the abdomen was rinsed with *n*-hexane. This suggests that the leader secretes the tandem running pheromone on abdominal surface, the compounds of which are soluble in *n*-hexane. This study sought to identify the glandular source of tandem running pheromone and its chemical structure in *Diacamma* sp. from Japan.

MATERIAL AND METHODS

Ant colonies

Five test colonies of *Diacamma* sp. from Japan, each comprised of one mated worker (gamergate), 56-120 unmated sterile workers and broods, were collected during 2007-2009 in Sueyoshi Park, Naha, on the main island of Okinawa, Japan. They were kept separately in small plastic cases (artificial nest, 110 × 75 × 25 mm, with a plaster floor) and placed at one end of a larger plastic container (foraging field, 640 × 380 × 90 mm). A red transparent

plastic sheet was placed on the artificial nests to keep them dark. Fresh water and mealworms were supplied every two days.

Inducing nest emigration

Nest emigration was artificially induced by removing the red plastic sheet and exposing the artificial nest to overhead (50 cm) light from a fluorescent lamp (100-W), as described by Traniello & Hölldobler (1984). Prior to that, another plastic case of the same size with a plastic sheet was placed at the opposite end of the foraging field as a new nest.

Behavioral bioassay

A total of 10 to 15 foragers from five colonies were individually killed by freezing, and their entire abdominal part, except for the petiole, were collected. These segments were used as dummies to imitate the leaders in the behavioral bioassay for tandem running.

After artificially inducing nest emigration, each isolated segment [I] was picked up with forceps and gently inserted between a leader and follower of a tandem pair and moved away from the former. We counted the numbers of ants that responded by following the dummy for at least five seconds. In the same way, we tested segments extracted with 1 ml of *n*-hexane for 5 min [II], as well as ones that had been re-treated with hexane extract [III]. We conducted this bioassay sequentially with [I], [II] and [III] at least seven times using different tandem pairs. The recovery effect of samples re-treated to *n*-hexane extracted abdominal segment was used to evaluate its tandem following activity.

The exocrine gland of the tandem running pheromone

To identify the glandular sources of the tandem running pheromone, we dissected three major exocrine glands - the Dufour's gland, poison gland and pygidial gland - from ten workers, and immersed each one in 500μl of *n*-hexane for 5 min. Each glandular extract was concentrated to an adequate volume, from which 0.1 ant equivalents (AE) each were tested. The bioassay was also conducted sequentially as described above at least 12 times using different tandem pairs.

Extraction and fractionation of tandem following pheromone

Thirty Dufour's gland extracts were chromatographed on 1 g of silica-gel (230-400 mesh, Merck) and successively eluted with 3 ml each of *n*-hexane

and dichloromethane (apolar and polar fraction). The *n*-hexane eluate was further chromatographed on 1 g of silica-gel (230-400 mesh, Merck), impregnated with 10% $AgNO_3$, and successively eluted with 3 ml of *n*-hexane and 15 % diethylether-i*n*-hexane (E/H) to separate saturated hydrocarbons and unsaturated hydrocarbons, respectively. All these fractions were used in the behavioral bioassays as test samples.

Chemical analysis

Active fractions were subjected to analysis with a Shimadzu GC-14A gas chromatograph equipped with a DB-1HT apolar capillary column (15 m × 0.25 mm ID × 0.1 μm film thickness, J&W Scientific), and a flame ionization detector (FID). Injection was made by splitless mode at 250°C for 1 min. Helium was used as the carrier gas at a column head pressure of 200 kPa. The GC oven temperature was held at 80°C for 1 min, increased from 80°C to 280°C at a rate of 20°C per min, and then held at the final temperature for 5 min. FID was set at 250°C.

Major qualitative components in these active fractions were analyzed by a Shimadzu GC-MS system QP-5000. A polar capillary column (DB-WAX, J&W Scientific: 30 m × 0.25 mm ID × 0.25 μm film thickness) was directly coupled to the mass spectrometer, and the carrier gas was helium at a column head pressure of 100 kPa. Injection was made by splitless mode at 230°C for 1 min. The GC oven temperature program was held at 60°C for 1 min, increased from 60 to 160°C at a rate of 10°C per min, and held at the final temperature for 5 min. The 70eV EI spectra were recorded at a rate of 0.5 s per scan.

Determination of pheromone structure

To determine the double bond position, the unsaturated hydrocarbon fraction was epoxydized with *m*-chloroperbenzoic acid (Swern, 1953). The resultant epoxides were analyzed by GC-MS. The mass spectra of the epoxides demonstrated characteristic diagnostic ions, so we could determine the position of the double bond.

E- and *Z*- isomers (contributed by Dr. Tetsu Ando of the Tokyo University of Agriculture and Technology) were used to determine the geometric configuration of the active monoenyl hydrocarbons. These isomers were also used in the behavioral bioassay as test samples.

RESULTS

Glandular source of tandem following pheromone

During tandem running, followers continuously orientated to the abdomens of the leaders. When isolated intact abdominal segments of leaders were placed within pairs of ants, they were followed in 17 of 20 trials (85%) (Fig. 1). After extracting the abdominal segment with *n*-hexane, however, only 5 of 20 ants (25%) followed the extracted segment. In contrast, 14 of 20 followers (70%) followed the segment when the extract had been re-applied. The number of ants that followed the segments extracted with *n*-hexane was significantly smaller than that for intact segments and those that had been re-treated with extract (Fisher's exact test with Bonferroni correction for multiple testing, $P<0.016$).

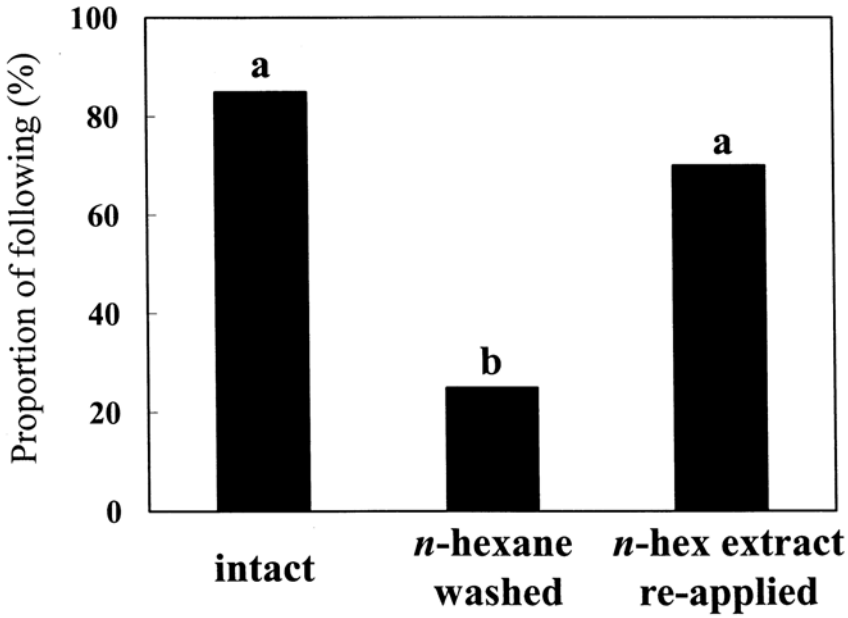

Fig.1. Proportion of the ants following intact, *n*-hexane extracted and re-treated *n*-hexane extract abdomens (n=15 for each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$).

Fig. 2 compares the tandem running responses to Dufour's gland, poison gland and pygidial gland. When testing the Dufour's gland extract, 12 of 15 (80.0%), 3 of 15 (20.0%) and 10 of 15 (66.7%) followers kept following the segments which were intact, extracted and treated with the Dufour's gland extract, respectively (Fisher's exact test with Bonferroni correction for multiple testing, $P<0.016$). In contrast, 13 of 16 (81.3%), 6 of 12 (50%) and 7 of 21 (33.3%) followers kept following the segments that were intact, extracted and treated with pygidial gland extract, while 14 of 16 (87.5%), 4 of 15 (26.7%) and 3 of 12 (25%) followers kept following the segments that were intact, extracted and treated with the poison gland extract, respectively. Neither pygidial gland nor poison gland extract had higher numbers of followers compared to extracted segments (Fisher's exact test with Bonferroni correction for multiple testing).

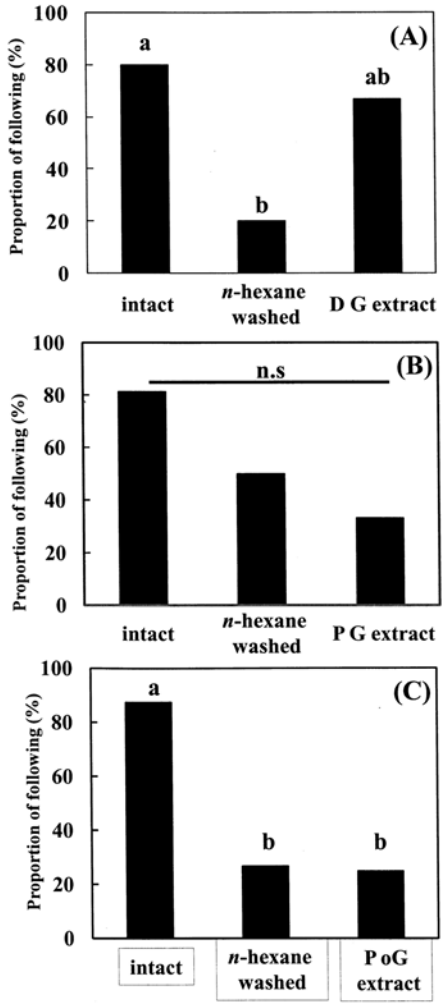

Fig.2. (A) Proportion of ants following intact, *n*-hexane extracted and re-treated Dufour's gland (DG) extract abdomens (n=15 each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$) (B) Proportion of ants following intact, n-hexane extracted and re-treated pygidial gland (PG) extract abdomens (n=16, 12, 21 for each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$). (C) Proportion of ants following intact, *n*-hexane extracted and re-treated poison gland (PoG) extract abdomens (n=16, 15, 15 for each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$).

Identification of the tandem running pheromone

Tandem running activity was compared between apolar and polar fractions obtained from the Dufour's gland extract (Fig. 3). The apolar fraction induced tandem running much the same as intact abdomens (Fisher's exact test with Bonferroni correction for multiple testing, $P<0.016$); 25 of 32 (78.1%) followers kept following the intact segment, and 4 of 27 (14.8%) and 31 of 54 (57.4%) followers kept following the extracted and treated ones, respectively. The polar fraction of Dufour's gland extract treated abdomen showed less activity. Ants followed 19 of 49 polar-fraction treated abdomens (38.8%), 25 of 32 intact ones (78.1%) and 1 of 20 *n*-hexane extracted abdomens (5.0%). We also checked the additive or synergistic effect of the polar fraction to the apolar fraction's activity, however, no significant effect was confirmed. Ants followed 9 of 15 apolar-fraction treated abdomens (60%), 10 of 15 re-mixed apolar- and polar-fraction treated abdomens (66.7%), 26 of 30 intact abdomens (86.7%) and 8 of 30 *n*-hexane extracted abdomens (26.7%).

Hydrocarbon compounds in the apolar fraction were further separated into saturated and unsaturated hydrocarbons to confirm their tandem running activity (Fig. 4). For the unsaturated hydrocarbon fraction, resultant tandem running activity was confirmed to be much the same as that of intact abdomens (Fisher's exact test with Bonferroni correction for multiple testing). Ants followed 12 of 20 intact abdomens (60.0%), 3 of 20 *n*-hexane extracted ones (15%) and 13 of 20 unsaturated-fraction treated ones (65.0%). The saturated hydrocarbon-fraction treated abdomen showed less tandem running activity. Ants followed 7 of 8 intact abdomens (87.5%), 1 of 7 *n*-hexane extracted ones (14.3%) and 2 of 7 saturated hydrocarbon-fraction treated ones (28.6%).

The unsaturated hydrocarbon fraction contained one major quantitative peak compound (X), for which the Kovats' retention index was 1667 on an apolar capillary column, and several minor compounds (Fig. 5-A). The mass spectrum of compound X showed a typical alkene fragmentation (*m/z* 69, 55 and 41 as base peak ion), and presented a molecular ion at m/z 238. We therefore estimated it as heptadecene ($C_{17:1}$). The epoxidized compound X presented the retention index as 1850 on an apolar capillary column, and gave two characteristic fragment ions at *m/z* 141 and *m/z* 155 (Fig. 5-B). This indicated the position of the double bond to be between the 8^{th} and 9^{th} carbon, and the compound X was identified as 8-heptadecene.

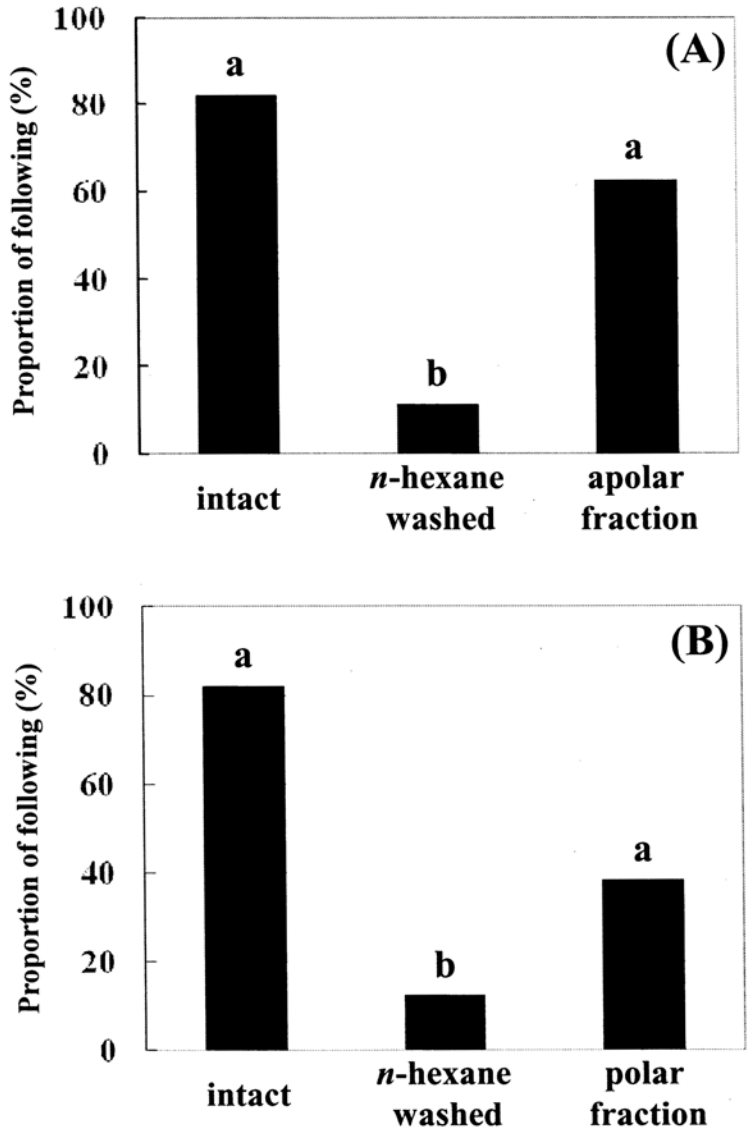

Fig.3. (A) Proportion of ants following intact, n-hexane extracted and re-treated apolar fraction of Dufour's gland extract abdomens (n=32, 27, 54 for each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing (P<0.016).(B) Proportion of ants following intact, *n*-hexane extracted and re-treated saturated hydrocarbon fraction abdomens (n=8, 7, 7 for each test)

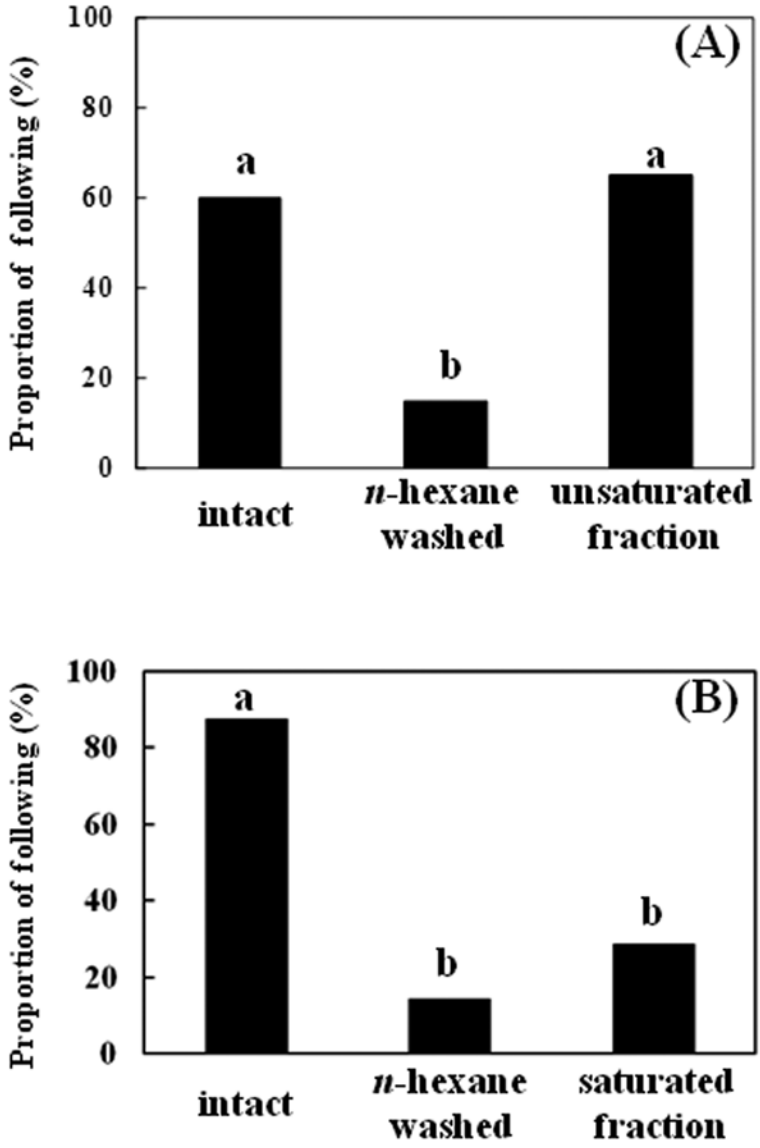

Fig.4. (A) Proportion of ants following intact, *n*-hexane extracted and re-treated unsaturated hydrocarbon fraction abdomens (n=20 for each test). (B) Proportion of ants following intact, *n*-hexane extracted and re-treated saturated hydrocarbon fraction abdomens (n=20 for each test). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$).

Fig. 6 shows tandem running activity of the synthesized (*E*)- and (*Z*)- isomer of 8-heptadecene. Ants followed only the (*Z*)- isomer of 8-heptadecene (Fisher's exact test with Bonferroni correction for multiple testing, $P<0.016$). Ants followed 9 of 10 intact abdomens (90.0%), 2 of 10 extracted ones (20.0%) and 6 of 10 (*Z*)-8-heptadecene treated ones (60.0%). The (*E*)-isomer of 8-heptadecene did not possess tandem running activity. Ants followed 5 of 5 intact abdomens (100%), 2 of 14 extracted ones (14.3%) but 0 of 5 polar-fraction treated ones (0%). (*Z*)-8-heptadecene was identified as the tandem running pheromone of *Diacamma* sp. from Japan.

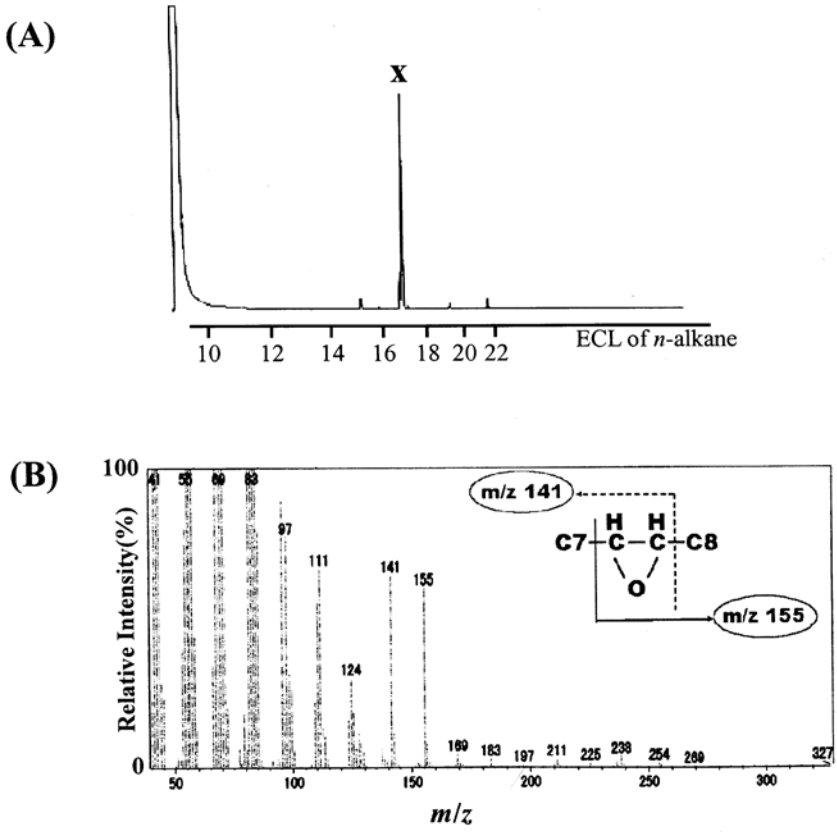

Fig.5. (A) Gas chromatogram of unsaturated hydrocarbon fraction from Dufour's gland . (B) Mass spectrum of peak x. Important fragment ions were observed at *m/z* 141 and *m/z* 155.

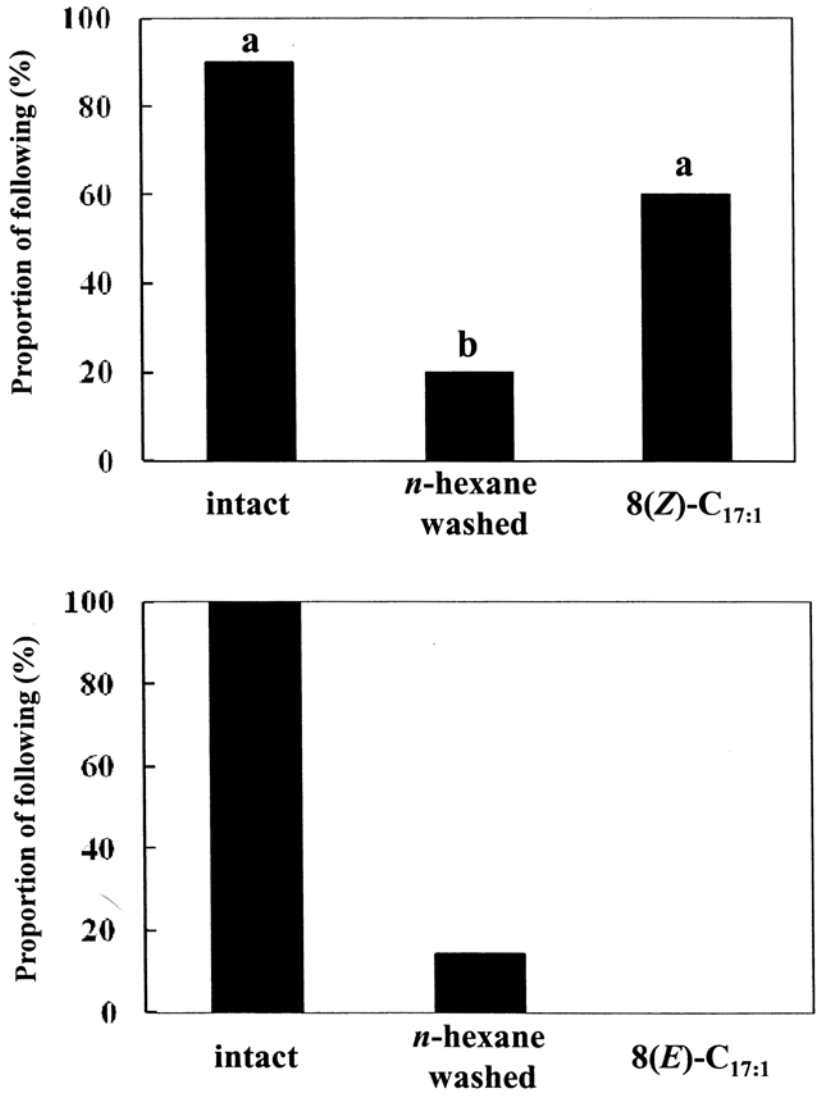

Fig.6. Proportion of ants following intact, *n*-hexane extracted and re-treated *E*- and *Z*- isomer of 8-heptadecene abdomens (n=10 for each test of *E*-isomer, n=5, 14 and 5 for *Z*- isomer). Bars with the same letter are not significantly different by Fisher's exact test with Bonferroni correction for multiple testing ($P<0.016$).

DISCUSSION

We confirmed that tandem running behavior of follower ants was mediated by chemical compounds on the abdominal surface of leader ants, and that the compounds are soluble in *n*-hexane. The glandular source of this chemical bond between the tandem pair was identified as the Dufour's gland. The most potent activity was confirmed from the apolar fraction of this gland; no additive or synergistic effect was observed from the polar fractions. The chemical structure of tandem running pheromone was revealed to be unsaturated hydrocarbon, and analytical data revealed the quantitatively major component of the unsaturated hydrocarbon fraction as 8-heptadecene. Synthesized (*Z*)-isomer of 8-heptadecene possessed tandem running activity equal to that of the isolated abdomen, but no attractive effect was observed when the abdomen was treated by *E*-isomer. We identified the tandem running pheromone of this ant as (*Z*)-8-heptadecene as it contributed greatly to the tandem running activity. Some possibility of the contribution of minor components remains. Some reports have already revealed that particular exocrine glands or organs such as poison gland, pygidial gland and hind legs, secrete tandem running pheromone (Möglich 1974; Hölldobler & Traniello 1980b; Maschwitz *et al.* 1986), but the active compound had not been identified. This is the first identification report of the tandem running pheromone of ants that is confirmed with the use of synthetic chemical compound that induced tandem running behavior.

Almost all recruitment pheromones of ants are reported to be derived from abdominal exocrine glands, including the Dufour's gland. In the subfamily Ponerinae, it is the poison gland and pygidial gland that have been identified as the source of tandem running pheromone (Möglich 1974; Traniello & Hölldobler 1984). *Diacamma* sp. from Japan belongs to the Ponerinae, but the source of tandem running pheromone in this species is Dufour's gland. During our bioassays, there was no characteristic behavior observed when the follower ant was presented the abdominal part treated with pygidial gland extract. However, when the followers were presented an abdominal part treated with poison gland extract, they showed aggressive or escape behavior. These two exocrine glands do not contribute to communication related to tandem running.

It is interesting that all the compounds so far identified as pheromone in the Dufour's gland belong to the terpenoid class; (Z, *E*)-α-farnesene (Solenopsis invicta, Vander Meer *et al.* 1988), all-trans-geranylgeranyl acetate and geranylgeraniol (*Ectatomma ruidum*, Bestmann *et al.* 1995) and a mixture of 4-methylgeranyl esters of fatty acids identified as trail pheromone (*Gnamptogenys striatula*, Blatrix *et al.* 2002). (*Z*)-8-Heptadecene is the first example of non-terpenoid recruitment pheromone secreted by the Dufour's gland. Compared to other already reported recruitment pheromones, the polarity of (*Z*)-8-heptadecene is low, because it does not include any polar function group in its structure. During the bioassay, we observed the characteristic behavior of the leader's application of the pheromone on the surface of their abdomen with their hind legs. Their body surfaces are known to be covered with cuticular hydrocarbons (CHCs) (Howard 1993). Both CHCs and tandem running pheromone are apolar, so are expected to have more affinity with each other than to the other reported recruitment pheromones that are mainly polar (Morgan 2009). This chemical nature of (*Z*)-8-heptadecene might contribute to the endurance of the effect of tandem running pheromone.

In most ant species, the recruitment pheromone is secreted from a single exocrine gland, but multiple glands may be involved in some cases (Vander Meer *et al.* 1998). In *Diacamma* sp. from Japan, only Dufour's gland possessed activity, however, we should confirm whether other exocrine glands possess additional and/or synergic effect.

It has been suggested that tandem running behavior could serve as a preadaptation for the evolution of chemical mass recruitment (Möglich *et al.* 1974). This is because it engenders movement of only a single ant at a time, and requires only a tandem running pheromone for the tandem pair. No orientation pheromones are needed. Other much more complex recruitment systems use sustainable chemical trails, and can attract many other workers to a food source, new nest site, or attack of intruders (Vander Meer *et al.* 1981; Vander Meer *et al.* 1988; Vander Meer *et al.* 1990; Hölldobler & Wilson 1978). Recruitment and orientation pheromone components and use of those pheromones differ in species. Detailed analysis of differences in recruitment systems among ants could reveal more about possible steps in the evolution of ant recruitment systems.

ACKNOWLEDGMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Areas, "Emergence of Adaptive Motor Function through Interaction between Body, Brain, and Environment," from the Japanese Ministry of Education, Culture, Sports, Science and Technology. This work was supported in part by grants-in-aid from the Japan Ministry of Education, Science and Culture (20033015, 21247006 to K. Tsuji).

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