Identification of sex pheromones of four economically important species in genus *Dendrolimus*

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Abstract **The female-produced sex pheromone of *Dendrolimus superans* was identified by gas chromatography (GC), coupled GC-mass spectrometry (GC-MS), electroantennographic (EAG) studies and field tests as a blend of (*Z*,*E*)-5,7-dodecadienal (*Z*5,*E*7-12:Ald) and (*Z*,*E*)-5,7-dodecadien-1-ol (*Z*5,*E*7-12:OH). In *D. kikuchii,* (*Z*,*E*)-5,7-dodecadien-1-yl acetate (*Z*5,*E*7-12:OAc) and *Z*5,*E*7-12:OH were found by GC and GC-MS analyses. However, in EAG studies male antennae were more sensitive to *Z*5,*E*7-12:OAc and (*Z*,*E*)-5,7-dodecadien-1-yl propionate (*Z*5,*E*7-12:OPr) than *Z*5,*E*7-12:OH. For *D. spectabilis*, *Z*5,*E*7-12:OH had been previously reported as the sex pheromone. However, in our studies, traps baited with *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr in a ratio of 1:1:1 caught three times more males than those baited with *Z*5,*E*7-12:OH alone. Relatively strong EAG responses were elicited from male antennae by *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr, but in addition to *Z*5,*E*7-12:OH, only very small amounts of *Z*5,*E*7-12:OAc was found in the pheromone gland. These facts suggest that *Z*5,*E*7-12:OAc is a pheromone minor components and *Z*5,*E*7-12:OPr is a sex attractant in *D. spectabilis*. For *D. tabulaeformis, Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr were found in extracts of pheromone glands of female moths, and the three compounds elicited strong EAG responses from antennae of male moths. These three compounds have been reported as a sex attractant of *D. tabulaeformis*, and our data confirm their roles as components of the sex pheromone of this species. The role of blend components in antagonizing cross attraction between congeners is discussed.**

Keywords: sex pheromone, *Dendrolimus superans*, *D. kikuchii*, *D. spectabilis*, *D. tabulaeformis*.

Among the 27 species in the genus *Dendrolimus* (Lepidoptera: Lasiocampidae) in China, the 6 species, *D. punctatus*, *D. superans*, *D. tabulaeformis*, *D. houi*, *D. spectabilis*, and *D. kikuchii* frequently appear in outbreak populations, causing widespread defoliation of their pine tree hosts. The biology, distribution, monitoring and control of these species have been studied extensively because of their economic importance[1—3].

To date, sex pheromones have been identified only in three species in this genus, *D. pini*, *D. spectabilis* and *D. punctatus*. The sex pheromone of *D. punctatus* was identified as a mixture of *Z*5,*E*7-12:OH and the corresponding acetate and propionate[4]. The sex pheromone of *D. spectabilis* from Japan was identified as *Z*5,*E*7-12:OH[5], but its attractiveness has not been tested with Chinese populations of this species. *Z*5,*E*7-12:Ald has been identified as the principal pheromone component in *D. pini*[6]. In addition to these sex pheromones, a sex attractant blend has been formulated for *D. tabulaeformis*, consisting of a mixture of *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr[7]. The common theme for all sex pheromone or sex attractant components identified so far in this genus is the *Z*5,*E*7-dodecadiene skeleton, with different terminal functional groups.

Our goal was to identify sex pheromones of other economically important species in the genus *Dendrolimus* in order to provide tools for monitoring their population densities. The identification of sex pheromones in these congeneric species also enabled us to determine the role of sex pheromone components in reproductive isolation between these species. Here, we report the identification of sex pheromone components for *D. superans*, *D. tabulaeformis*, *D. kikuchii*, as well as minor pheromone components in *D. spectabilis*.

1 Materials and methods

(ⅰ) Insects. Cocoons of *D. tabulaeformis* were collected from the host tree, *Pinus tabulaeformis*, in Beijing; cocoons of *D. kikuchii* were collected from the host tree, *P. massoniana,* in Dongzhi County, Anhui Province, and cocoons of *D. superans* were collected from the host tree, *Larix gmelin*, in Xifeng County, Liaoning Province. Third instar larvae of *D. spectabilis* were collected from Fuxin County of Liaoning Province, and reared on foliage of the host tree, *P. tabulaeformis*. All insects were maintained in a rearing cabinet with a reversed photoperiod of 16L:8D, 24—26℃ and 60%—80% relative humidity. Newly emerged moths were sexed and maintained under the same conditions as used for mass rearing.

(ⅱ) Preparation and purification of chemicals. *Z*5,*E*7-12:OAc was purchased from Chemtech B.V. (Amsterdam, The Netherlands), and (*Z*)-5-dodecenyl acetate (*Z*5-12:OAc) was purchased from Jiangsu Hormone Institute (Jintan City of Jiangsu Province, China). *Z*5,*E*7-12:OH and *Z*5-12:OH were prepared from the corresponding acetates by transesterification in 0.5 mol/L KOH-methanol, and *Z*5,*E*7-12:OPr by propionation of *Z*5,*E*7-12:OH with propionyl chloride. *Z*5,*E*7-12:Ald and *Z*5-12:Ald were prepared from the corresponding alcohols by oxidation with pyridinium chlorochromate[8]. All chemicals were purified by preparative GC using a 2 m×3.2 mm column (packed with 8% PEG 20 M on 80—100 mesh Chromosorb G-HP) in a Model 990 Gas Chromatograph (Perkin-Elmer). The purities of all purified compounds were determined by analysis on a BP-20 cap-

illary column (50 m×0.22 mm ID, 0.25-μm film, Scientific Glass Engineering Pty. Ltd., Australia) with a 5890 Series Ⅱ Gas Chromatograph (Hewlett-Packard). All chemicals used for EAG analyses were ≥99% chemically and isomerically pure. Compounds used for field trials were ≥99% in chemical purity and ≥96% in isomeric purity. Reference compounds, *ZE*, *EZ*, *ZZ* and *EE*-12:OH were from our laboratory collection.

(iii) Preparation and analysis of pheromone gland extracts. Pheromone glands of calling females (1—2 days old) 4—7 h after scotophase were excised and extracted for approx. 30 min in 10 μL distilled hexane. Analyses of extracts were conducted with a 5890 series Ⅱ Gas Chromatograph (Hewlett-Packard) in splitless mode with nitrogen carrier gas. Oven temperature were programmed as follows: BP-20 column, (50 m×0.22 mm ID) 80℃ hold for 1 min, 10℃/min to 200℃, hold for 30 min; BP-1 column, (50 m×0.22 mm ID) 80℃ hold for 1 min, 10℃/min to 230℃, hold for 40 min. GC-MS analyses were performed on a Finnigan Voyager mass spectrometer interfaced with a trace 2000 gas chromatography using a 25 m×0.22 mm ID BP-20 column programmed from 80℃ to 200℃ at a rate of 10℃/min.

(iv) Electroantennography (EAG). EAG response profiles of male *D. superans*, *D. kikuchii*, *D. spectabilis*, and *D. tabulaeformis* to synthesized standards were recorded as described by Visser[9] and Cossé et al.[10]. A defined amount of test chemical in hexane was applied to a filter paper strip (3 cm×0.5 cm), which was placed inside a Pasteur pipette. Stimuli were introduced as a short puff (2 mL) into a purified, humidified airstream from the Pasteur pipette, through a hole in the stainless steel tube positioned 9 cm from the antenna. The delivering puffs were ~0.1 s duration. Solvent blank puffs (filter paper plus solvent) served as controls. To compensate for possible deterioration of the antennal preparation, a standard compound, *Z*5-12:OAc, preceded each test stimulus puff. Relative response of a test compound was expressed as a percentage relative to the response (100) to *Z*5-12:OAc.

(v) Field trials. Test chemicals were dissolved in hexane, and then loaded onto gray rubber septa (The West Company, Phoenixville, Pa, USA) at a dose of 500 μg. Once the solvent had evaporated, two 100 μL aliquots of dichloromethane were added to the septum to help transport any residual pheromone into the septa. Sticky traps, similar in shape to the Pherocon 1C traps from Trece Inc. (Salinas CA, USA), were constructed from two pieces of cardboard (42 cm×28 cm). Traps were hung on the branches of pine trees ca. 2 m above ground, at a spacing of at least 15 m between traps in a complete block design. Trap positions were randomized within a replicate to minimize the effects of habitat heterogeneities. Moth catches were recorded daily and then traps were rerandomized each day.

(vi) Statistical analysis. For the statistical analysis of field trials, day effect was eliminated by pooling the daily trap counts for each treatment in a given block. This sum (x) were regarded as replicates and statistically analyzed after transformation to log (x + 1), and then subjected to a one-way ANOVA. If the F value was significant, differences between treatment means were tested for significance by Student-Newman-Keuls (SNK, $\alpha = 0.05$) test (SAS/STAT User's Guide, 1988, release 6.03 edition, SAS Institute).

2 Results

(i) *D. Superans*. Components in the pheromone gland extracts were identified by comparison of retention times to those of reference compounds on both polar (BP-20) and nonpolar (BP-1) GC columns, and by comparisons of mass spectra. *Z*5,*E*7-12:Ald, *Z*5,*E*7-12:OH, *Z*5-12:Ald and *Z*5-12:OH were found in a ratio of 100∶98∶69∶80 in the gland extracts (table 1).

All four geometric isomers of 5,7-dodecadiene and two geometric isomers of 5-dodecene can be separated by both polar and nonpolar columns, so that dienes were confirmed to have the *Z*5,*E*7 configuration, and the monoenes were determined to have *Z*5 configuration. The mass spectra of the four female-produced components matched those of the corresponding synthetic compounds. In EAG studies, male antennae of *D. superans* were most sensitive to *Z*5,*E*7-12:Ald, followed by *Z*5,*E*7-12:OH (fig. 1, *D. superans*), with weaker responses elicited by *Z*5-12:OH and *Z*5-12:Ald.

Based on the results from chemical and EAG analyses, baits with different combinations of pheromone candidates were prepared (table 2). In test 1, only a few males were captured because the test began at the end of the flight season. However, the field data suggested that baits containing *Z*5,*E*7-12:Ald and *Z*5,*E*7-12:OH might be most. In test 2, all traps baited with the two diene components *Z*5,*E*7-12:Ald and *Z*5,*E*7-12:OH caught statistically equal numbers of males. It appeared that *Z*5-12:Ald and *Z*5-12:OH might enhance trap catches but this remains to be determined in further tests. Removal of either one of the diene components reduced trap captures, demonstrating that the two dienes were required components.

(ii) *D. kikuchii*. GC analysis of pheromone gland extracts on both BP-20 and BP-1 column showed the presence of *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH in a ratio of 100∶22 (table 1). *Z*5,*E*7-12:OPr was not detected, nor were other isomers of the diene acetates found. The identity of the female-produced *Z*5,*E*7-12:OAc was confirmed by GC-MS analysis with reference compound. It was not possible to obtain a full-scan mass spectrum for *Z*5,*E*7-12:OH due to its small amounts and coeluting compounds in the extract analyzed. However, the diag-

nostic ions of this compound, *m*/*z* 182 (M^+), 164 (M^+-H_2O) were found in the extract. These results were further confirmed by acetylation and methanolysis of the gland extracts. After acetylation, the peak with the same retention time as *Z*5,*E*7-12:OH in the gland extract disappeared. After methanolysis of the acetylazed sample, a relatively large peak with the same retention time as *Z*5,*E*7-12:OH reappeared, and the peak with the same retention time as *Z*5,*E*7-12:OAc disappeared. EAG trials showed that the male antennae of *D. kikuchii* were most sensitive to *Z*5,*E*7-12:OAc, followed by *Z*5,*E*7-12:OPr, *Z*5-12:OAcand *Z*5,*E*7-12:OH, with low responses to other test compounds (fig. 1, *D. kikuchii*).

(iii) *D. spectabilis*. A number of baits containing possible pheromone related compounds were prepared in order to test the attractiveness of *Z*5,*E*7-12:OH alone and in combination with related compounds. Field tests showed that the traps baited with *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr in a 1∶1∶1 ratio caught approx. 3 times more males than that baited with

Table 1 Relative proportions of components identified from single female extracts of *D. tabulaeformis*, *D. kikuchii*, *D. spectabilis* and *D. superans*[a)]

Compound	Proportion relative to the most abundant component in each species (Mean±SD)			
	D. tabulaeformis N = 7	*D. Kikuchii* N = 17	*D. spectabilis* N = 10	*D. superans* N = 13
Z5,E7-12:OPr	29±17.8	–	–	–
Z5,E7-12:OAc	100	100	3.2±1.8	–
Z5,E7-12:OH	47±36.5	22.4±19.1	100	97.9±72.9
Z5,E7-12:Ald	–	–	5.6±5.4	100
Z5-12:OH	–	–	–	80.1±63.6
Z5-12:Ald	–	–	–	68.7±74.6

a) GC retention time matches on two columns (see methods and materials) with authentic standard except for *D. tabulaeformis*, in which the extracts were analyzed by BP-20 column. –, not detected in the female extracts.

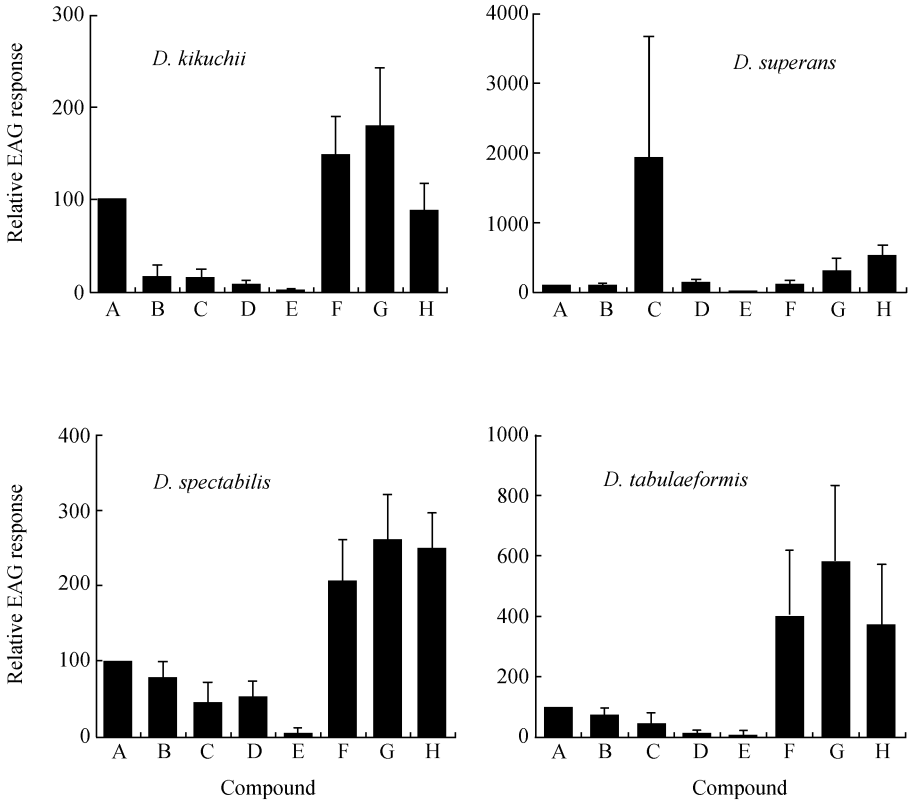

Fig. 1. Relative EAG responses (mean+SD) of male *D. kikuchii* (N = 24), *D. superans* (N = 16), *D. spectabilis* (N = 22) and *D. tabulaeformis* (N = 14) to compounds identified from the sex pheromone gland of *Dendrolimus* spp. and related compounds at a dose of 10 μg. A = *Z*5-12:OAc; B = *Z*5-12:OH; C = *Z*5,*E*7-12:Ald; D = *Z*5-12:Ald; E = 12:Ald (dodecadienal); F = *Z*5,*E*7-12:OPr; G = *Z*5,*E*7-12:OAc; H = *Z*5,*E*7-12:OH.

Table 2 Male captures of *D. superans* in traps with different combinations of components found in the pheromone gland extracts

Composition of baits/μg[a)]				Total catch	
*Z*5,*E*7-12:Ald	*Z*5,*E*7-12:OH	*Z*5-12:Ald	*Z*5-12:OH	Test 1[b)]	Test 2[c)]
500	–	–	–	1	13 ab
–	500	–	–	0	3 a
272.7	227.3	–	–	3	34 bc
200	167	133	–	5	35 bc
187.5	156.25	–	156.25	1	31c
300	–	200	–	0	24 bc
–	250	–	250	0	0
150	125	100	125	2	51c
–	–	–	–	0	5 a

a) The ratios of the components in baits were similar to that found in the pheromone gland. b) the experiments were conducted in Xifeng County, Liaoning Province (4 replicates over 2 nights). c) The experiments were conducted in Weichang County, Hebei Province (4 replicates over 5 nights). Numbers followed by the same letter in each treatments were not significantly different at the 5% confidence level by SNK ($F = 8.92$, $df = 7,24$, $P<<0.001$).

*Z*5,*E*7-12:OH alone. The attractiveness of lures with *Z*5,*E*7-12:OH plus *Z*5,*E*7-12:OAc or *Z*5,*E*7-12:OPr was not significantly increased or decreased when compared with traps baited with *Z*5,*E*7-12:OH alone. However, baits containing *Z*5,*E*7-12:OH and *Z*5,*E*7-12:Ald in a ratio of 1∶1 resulted in significantly decreased trap captures as compared with virgin female or *Z*5,*E*7-12:OH alone (table 3).

Table 3 Catches of male *D. spectabilis* in traps baited with various combinations of *Z*5,*E*7-12:OH, *Z*5,*E*7-12:Ald, *Z*5,*E*7-12:OPr and *Z*5,*E*7-12:OAc

Composition of baits/μg				Total catch[a)]
*Z*5,*E*7-12:Ald	*Z*5,*E*7-12:OH	*Z*5,*E*7-12:OPr	*Z*5,*E*7-12:OAc	
–	500	–	–	30 bc
250	250	–	–	6 a
–	250	250	–	37 cd
–	250	–	250	14 ab
–	166.7	166.7	166.7	86 d
Virgin female				47 cd

a) The experiments were conducted in Fuxin County, Liaoning Province (4 replicates over 7 nights). Numbers followed by the same letter in each treatment were not significantly different at the 5% confidence level by SNK ($F = 12.56$, $df = 5, 18$, $P<<0.001$).

Gland extracts of *D. spectabilis* contained *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:Ald in a ratio of 100:3.2:5.6 (table 1). Other geometric isomers of *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OPr were not detected; however large EAG responses were elicited from male *D. spectabilis* by *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr, with weaker responses elicited by several other components (fig. 1, *D. spectabilis*).

(ⅳ) *D. tabulaeformis*. GC analysis of the female extracts indicated that *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OPr were detected in a ratio of 100:47:29 (table 1). EAG studies showed that *Z*5,*E*7-12:OAc elicited about six times larger response than *Z*5-12:OAc (fig. 1, *D. tabulaeformis*) whereas responses to *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OPr were intermediate.

3 Discussion

Both chemical and EAG analysis and field tests suggest that *Z*5,*E*7-12:Ald and *Z*5,*E*7-12:OH are major sex pheromone components of *D. superans*. Baits lacking either one of these compounds significantly decreased trap catches. No clear effect was found from addition of *Z*5-12:OH or *Z*5-12:Ald to baits, but more field tests should be performed to further elucidate their possible roles in the pheromone blend.

*Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH were found in the pheromone gland of *D. kikuchii* by GC, GC-MS and micro-chemical reaction. EAG analyses showed that *Z*5,*E*7-12:OAc elicited the largest response from antennae of male *D. kikuchii*. These results suggest that *Z*5,*E*7-12:OAc is a major pheromone component in *D. kikuchii*, but this remains to be clarified with field tests.

For *D. spectabilis*, field trials revealed strong synergism by adding *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr to baits containing *Z*5,*E*7-12:OH. In the pheromone gland, *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OAc were found in a ratio of 100∶3.2 (table 1), and *Z*5,*E*7-12:OAc also elicited strong EAG responses from males (fig. 1, *D. spectabilis*). Surprisingly, although *Z*5,*E*7-12:OPr has not found in gland extracts, it elicited strong EAG responses from males' antennae. Thus, both laboratory and field data suggest that *Z*5,*E*7-12:OAc is a pheromone minor component and *Z*5,*E*7-12:OPr is a sex attractant and could be minor sex pheromone components of *D. spectabilis*. However, only *Z*5,*E*7-12:OH had been reported previously as a sex pheromone component of *D. spectabilis*[5], possibly due to the very small amounts of *Z*5,*E*7-12:OAc and *Z*5,*E*7-

12:OPr in the pheromone gland.

The strong EAG responses to *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OPr by *D. tabulaeformis* antennae, and the fact that the three components also were found in the pheromone gland support previous work suggesting that its sex attractant consists of these three components [7]. This was further confirmed by a field test, which showed that traps baited with the three components in a ratio similar to that found in the pheromone gland caught significantly more males than traps baited with any single component or any combination of two components (data not shown).

D. punctatus, *D. tabulaeformis* and *D. spectabilis* use the same components, *Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr, as sex pheromone components. The similarity of the pheromonal composition in the three species suggests that they be closely related. To our knowledge, ratio of the three components could not be important for male capture in *D. punctatus*[11]. However, it still remains to study whether the ratio of the three components is important for male capture in *D. tabulaeformis* and *D. spectabilis*. It has been found that the pheromone receptor system of *D. punctatus* is composed of specialist cells for *Z*5,*E*7-12:OH, *E*5,*Z*7-12:OH, *Z*5,*E*7-12:OAc, *E*5,*Z*7-12:OAc and *Z*5,*E*7-12:OPr[6]. In addition, two monoenes, *Z*5-12:OH and *Z*5-12:OAc, were found in the pheromone gland of *D. punctatus*. In field tests, baits containing both *ZE* and *EZ* isomers of acetate, alcohol and propionate plus the two monoenes caught significantly more males than those containing the three *ZE* isomers[11]. It remains to be determined whether the *EZ* isomers and/or the two monoenes have any role in the pheromone blends, and in reproductive isolation among these three species.

It is interesting to note that the major pheromone component of *D. superans* and *D. pini*, *Z*5,*E*7-12:Ald, apparently inhibits behavioral responses of *D. spectabilis* (table 3). *Z*5,*E*7-12:Ald also resulted in antagonistic effects on male trapping of *D. punctatus* when it was added to the pheromone blend (*Z*5,*E*7-12:OH, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OPr) in a ratio of 20∶80. However, when the ratio was changed to 5∶95, no effect was observed (Zhao, unpublished data). On the other hand, *Z*5, *E*7-12:OAc, the pheromone component in *D. punctatus*, is a powerful inhibitor of the sex pheromone of *D. pini*[6]. This suggests that the *Dendrolimus* species could be divided into two types according to their pheromonal composition. The first type (*D. tabulaeformis*, *D. spectabilis*, and *D. punctatus*) uses *Z*5,*E*7-12:OAc as a pheromone component, whereas the second type (*D. superans* and *D. Pini*) uses *Z*5,*E*7-12:Ald as a pheromone component. *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:Ald may be the factors for reproductive isolation between the two types of species.

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