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Ecological, morphological and molecular studies on *Ganaspis* individuals (Hymenoptera: Figitidae) attacking *Drosophila suzukii* (Diptera: Drosophilidae)

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Running title: *Ganaspis* parasitoids of *Drosophila suzukii*

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Abstract

Ganaspis individuals parasitizing *Drosophila suzukii* (Matsumura), a pest of fruit crop, were examined for host use and molecular and morphological differences from those attacking *D. lutescens* Okada and some other *Drosophila* species that breed on fermenting fruits. Wild cherry fruits were collected in the suburbs of Tokyo, and drosophilid pupae obtained from these fruits were examined for parasitism. *Drosophila suzukii* was the only drosophilid species infesting fresh wild cherry fruits, and *Ganaspis* individuals were the major parasitoids attacking *D. suzukii* in wild cherry fruits. In parasitism experiments, these *Ganaspis* individuals parasitized *D. suzukii* larvae in fresh cherry fruits, but did not parasitize those in Drosophila medium. In addition, they did not parasitize larvae of some other fruit-feeding *Drosophila* species even when these occurred in fresh cherry fruit. These *Ganaspis* individuals parasitizing *D. suzukii* were different from those parasitizing *D. lutescens* and some other drosophilids in nucleotide sequences of the COI gene, as well as in ITS1 and ITS2. They were also different in the forewing and antenna morphology, although they showed some overlap in the morphological traits. They are tentatively assigned as the *suzukii*- and *lutescens*-associated types of *G. xanthopoda* (Ashmead). In the present field survey, *Leptopilina japonica* Novković & Kimura and some *Asobara* species were also observed to attack *D. suzukii* larvae in wild cherry fruit.

Keywords

Drosophila – Host use – Morphology – Nucleotide sequence –Parasitoids

Introduction

Drosophila suzukii (Matsumura) is a pest of soft fruit such as cherry, strawberry or blueberry. Females of this species lay eggs in ripening fruit before harvest and cause serious damage (Kanzawa 1939; Kimura et al. 1977; Nishiharu 1980; Mitsui et al. 2006, 2010; Lee et al. 2011; Walsh et al. 2011; Calabria et al. 2012), unlike many other fruit-feeding *Drosophila* species that lay eggs in damaged or rotting fruit. This species has its origin in the temperate and subtropical regions of Asia (Kanzawa 1939; Lemeunier et al. 1986), and has recently colonized North America and Europe (Hauser 2011; Walsh et al. 2011; Calabria et al. 2012). For development of integrated management system of this pest species, information on the natural enemies is very important (Walsh et al. 2011; Chabert et al. 2012). So far, *Trichopria* sp. (Hymenoptera, Diapriidae; cited as *Phaenopria* sp. in Kanzawa's paper), *Asobara japonica* Belokobylskij, *A. tabida* Nees von Esenbeck (Hymenoptera, Braconidae) and *Ganaspis xanthopoda* (Ashmead) (Hymenoptera, Figitidae) have been reported to parasitize some frugivorous *Drosophila* species including *D. suzukii* (Kanzawa 1939; Mitsui et al. 2007; Ideo et al. 2008). In our parasitism experiments (Mitsui and Kimura 2010), however, *Ganaspis* individuals that emerged from *D. lutescens* Okada (a species breeding on fermenting fruit) and were identified as *G. xanthopoda* did not parasitize *D. suzukii*, suggesting that the *Ganaspis* individuals parasitizing *D. suzukii* and *D. lutescens* are different host races or host-specific species. To verify this possibility, we carried out field survey and parasitism experiments on *Ganaspis*

individuals parasitizing *D. suzukii* and examined the molecular and morphological differences between individuals parasitizing *D. suzukii* and *D. lutescens*.

Materials and methods

Field survey

Wild cherry fruit (*Prunus donarium* Sieb.) is one of major resources of *D. suzukii* in late spring and early summer in central Japan (Nishiharu 1980; Mitsui et al. 2006, 2010). This fruit (about 5 mm in diameter) changes color from yellow to black on tree and then falls on ground. *Drosophila suzukii* females mainly lay eggs in black fruits before fall (Mitsui et al. 2006). In the present survey, black fruits on trees and those soon after fall on the ground were collected in wooded areas in Naganuma park located in the suburbs of Tokyo (35.6 °N, 139.4 °E) in June, 2010 and 2011. In 2010, fruits were collected from three sites (sites A, B and C) in this park, while from various sites in 2011. Collected fruits were brought back to the laboratory and placed in plastic containers with cloth or paper. When drosophilid larvae in the fruits pupated on cloth or paper, they were collected, identified to species, placed on Petri dishes with wet filter paper, and examined for the emergence of flies or parasitoids. When parasitoids emerged, some were maintained alive in vials containing Drosophila medium to examine their host use and some were preserved in 100 % alcohol for molecular and morphological studies. In addition, *Ganaspis* individuals that emerged from *D. suzukii*

pupae collected from wild cherry fruits in Sendai (38.2 °N, 140.9 °E) and those that emerged from *D. lutescens* pupae collected from banana baits placed in wooded areas in the suburbs of Tokyo and Sendai were used for molecular and morphological studies.

Parasitism experiments

To examine the host specificity of *Ganaspis* individuals, parasitism experiments were conducted using the following *Drosophila* species, *D. suzukii*, *D. lutescens*, *D. rufa* Kikkawa & Peng, *D. auraria* Peng, *D. biauraria* Bock & Wheeler and *D. triauraria* Bock & Wheeler. These *Drosophila* species belong to the *melanogaster* species group (Lemeunier et al. 1986), and are major native fruit-feeding species in central Japan (Nishiharu 1980; Mitsui et al. 2010). Laboratory stocks of these *Drosophila* species originated from several females collected in and near Tokyo and were maintained on cornmeal-malt medium for a month to several years in laboratory. Parasitism experiments were performed as follows. Five to 20 females of these *Drosophila* species were introduced into a vial containing a cherry fruit (*Prunus avium* L.) and allowed to oviposit for a day. Intact cherry fruit was provided for *D. suzukii*, whereas fruit pricked by needle was provided for the other five species since they did not oviposit in intact fruits. Two days later, five *Ganaspis* females and several males were introduced to the vial and allowed to oviposit. After two days, parasitoids are removed from the vial and emergence of flies and/or parasitoids from the vial was recorded. In addition, *Ganaspis* females were examined whether they parasitize *D. suzukii* larvae

growing in cornmeal-malt medium (Drosophila medium) or not. Experiments were carried out in the same was as described above, except not cherry fruit but cornmeal-malt medium was provided. The experiments were done under a long-day condition (15 h light-9 h dark) at 23 °C.

Molecular analysis

Nucleotide sequences of mitochondrial COI (cytochrome oxidase subunit I) gene and two nuclear DNA regions, ITS1 (intertranscribed spacer sequence I of ribosomal RNA genes) and ITS2, were determined for *Ganaspis* individuals collected. DNA was extracted from each specimen by a modified phenol-chloroform protocol. All amplifications were preformed in 23μl reaction volumes containing 1.3 mM $MgCl_2$, 0.042 mM dNTP, 2.6 μM primers, 0.042 U Ampli *Taq* DNA polymerase, and 2.4 μL 10× PCR buffer. PCR profile consisted of one cycle of denaturation (94°C for 10 min), 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1.5min), followed by one cycle of final extension at 72°C for 12min. Amplified products were diluted to 1ng/μL, and used as sequencing templates.

Amplification of ITS1 and ITS2 fragments was performed by following primer pairs: 5’-GCTGCGTTCTTCATCGAC-3’ and 5’-CGTAACAAGGTTTCCGTAGG-3’ for ITS1 (412-647 bp); 5’-TGTCAACTGCAGGACACATG-3’ and 5’-AATGCTTAAATTTAGGGGGTA-3’ for ITS2 (448-564 bp). Amplification of COI fragments was preformed with a pair of

primers, 5’-GGTCAACAAATCATAAAGATATTGG-3’ (LCO) and 5’-TAAACTTCAGGGTGACCAAAAAATCA-3’ (HCO) (about 600 bp) (Folmer et al. 1994).

For all sequence reactions, Big Dye Terminator Cycle Sequencing Kit (ABI) was used. Sequencing was carried out with a 3100 Genetic Analyzer (ABI), utilizing the same primers used for PCR amplification.

Phylogenetic analysis of sequence data was conducted using Mega5 software (Tamura et al. 2011). Sequences were aligned manually and used to construct phylogenetic trees using neighbor-joining (NJ) method (Saitou and Nei 1987). Nucleotide distances in NJ trees were estimated by Kimura’s two-parameter method (Kimura 1980). In the phylogenetic tree with COI, *Leptopilina japonica japonica* Novković & Kimura and *L. heterotoma* (Thompson) were used as outgroup. Bootstrap values were obtained after 1000 replications.

Morphometric analysis

Forewing and antenna were subjected to the analysis. For forewing, geometric morphometric analysis was conducted with 169 females (61 from *D. suzukii* and 108 from *D. lutescens*) and 153 males (46 from *D. suzukii* and 107 from *D. lutescens*). The right forewing of each specimen was mounted on a slide in Hoyer's medium. Digitized forewings were assigned 7 landmarks, positioned at forewing vein intersections and terminations (Fig 1) using TpsDig 2.12 software (Rohlf 2008a). To

examine forewing size differences among wasps, ANOVA was conducted on the centroid size parameter (the square root of the sum of squared distances between each landmark and the forewing centroid). In order to extract shape variables, raw coordinates were first superimposed by a generalized orthogonal least-square Procrustes (GPA) procedure, standardizing the size of landmark configurations and removing translational and rotational differences (Rohlf and Slice 1990). Next, the partial warps were calculated, and the obtained 'weight matrix' (*w*; Rohlf et al. 1996) was subjected to canonical discriminant analysis. Centroid size and weight matrix were obtained utilizing TpsRelw 1.46 software (Rohlf 2008b).

In the antenna analysis, 140 females (52 from *D. suzukii* and 88 from *D. lutescens*) and 131 males (36 from *D. suzukii* and 95 from *D. lutescens*) were used. The digitized antenna of each specimen was measured for length of each segment using Photoshop CS5.1 (Adobe systems Incorporated, San Jose, USA). The proportion of each segment to total length was then calculated, and the obtained matrix was subjected to canonical discriminant analysis.

Statistical analyses were preformed using Jmp ver. 6.1 (SAS Institute, Cary USA).

Results

Field survey

In 2010, a total of 3,938 *D. suzukii* pupae were obtained from 3,892 wild cherry fruits collected; 367 pupae from 319 fruits on trees and 3,571 pupae from 3,573 fallen fruits (Table 1). In 2011, the numbers of fruits and pupae collected were not counted. *Drosophila suzukii* was the only species that bred on these fruits. In 2010, 26 *Ganaspis* individuals emerged from 367 *D. suzukii* pupae collected from fruits on (the parasitism rate was about 7%), and a total of 141 individuals emerged from 3,571 pupae collected from fallen fruits (the parasitism rate was about 4%). In 2011, 112 *Ganaspis* individuals emerged. In addition to *Ganaspis* individuals, *Leptopilina japonica japonica* and unidentified *Asobara* species were recorded from *D. suzukii* pupae collected from fallen fruits.

Parasitism experiments

Table 2 shows the results of parasitism experiments on *Ganaspis* individuals that emerged from *D. suzukii* pupae. In the experiment where *D. suzukii* was used as host, 129 *Ganaspis* individuals and 408 host flies emerged when cherry fruits were used, but no *Ganaspis* individuals emerged when Drosophila medium was used. In the experiment with the other five *Drosophila* species, no *Ganaspis* individuals emerged even when cherry fruits were used.

Molecular analysis

The ITS1 sequence was successfully obtained from 22 *Ganaspis* individuals out of 23 individuals used in the molecular analysis, whereas the COI and ITS2 sequences were obtained only from 12 and 15 individuals, respectively. Figure 2 shows the neighbor-joining phylogenetic trees based on these three nucleotide sequences. Irrespective of original locality (i.e., Tokyo or Sendai), *Ganaspis* individuals from *D. suzukii* and those from *D. lutescens* are discriminated by these sequences with high (>97%) bootstrap support. Nucleotide divergence between them ranged from 4.5 to 5.7% for COI, from 7.2 to 8.6% for ITS1, and from 1.7 to 2.6% for ITS2.

Morphological analysis

Figure 3 shows the distributions of canonical variate scores in the discriminant analyses on forewing shape and relative length of antennal segments of females and males. These traits were significantly different between *Ganaspis* individuals from *D. suzukii* and those from *D. lutescens* (ANOVA, $P<0.001$). Especially, the overlap of the canonical variate scores in the antenna analysis was little. However, the overlap in the forewing analysis was considerably large. Forewing centroid size (FC) and antennal length (AL) were significantly (ANOVA, $P<0.001$) larger in individuals from *D. suzukii* (FC=339.5±17.4 in the female and 316.9±19.9 in the male (mean±SD); AL=1.42±0.07 in the female and 2.36±0.16 in the male (mean±SD in mm) than in those from *D. lutescens* (FC=284.0±16.7 in the female and 272.4±16.0 in the male; AL=1.11±0.06 in the female and 2.00±0.12 in the male) in both sexes.

Discussion

Ganaspis individuals from *Drosophila suzukii* parasitized *D. suzukii* larvae occurring in fresh cherry fruit but did not those in Drosophila medium, and further they did not parasitize larvae of some other fruit-feeding *Drosophila* species even if they occurred in cherry fruit. The field survey revealed that they parasitize *D. suzukii* larvae in wild cherry fruit on trees, but it was not determined whether they also parasitize *D. suzukii* larvae in fallen fruits or not. In contrast, *Ganaspis* individuals from *D. lutescens* parasitize larvae of *D. lutescens, D. rufa, D. biauraria, D. triauraria* and some other species occurring in Drosophila medium, but they did not parasitize *D. suzukii* larvae occurring in Drosophila medium (Mitsui and Kimura 2010). Thus, *Ganaspis* individuals from *D. suzukii* and *D. lutescens* do not overlap in host use, and the former seems to be specializes to *D. suzukii*.

The *Ganaspis* individuals from *D. suzukii* and *D. lutescens* were clearly discriminated by nucleotide sequences of COI, ITS1 and ITS2 with high bootstrap value. In addition, they were statistically different in the morphology of forewing and antenna. These results suggest that the gene flow is limited between them. Although they might have diverged as different species, in the present paper, they are tentatively assigned as the *suzukii*-associated and the *lutescens*-associated types of *G. xanthopoda* (since *D. lutescens* is the major host of the latter). To determine their species status, difference from or identity with the holotype specimen must be examined. For this purpose,

however, morphological comparisons would not be sufficient, because the two types of *G. xanthopoda* reported in this study cannot be distinguished only by their morphology. It is necessary to examine the molecular differentiation and reproductive isolation of the two types of *G. xanthopoda* in this study from individuals occurring at the type locality, i.e., Grenada of the Caribbean region (Ashmead 1896). This remains for future study.

Trichopria sp., *Leptopilina japonica* and some *Asobara* species also parasitize *D. suzukii* as observed in the present and previous studies (Kanzawa 1939; Mitsui et al. 2007; Ideo et al. 2008), but they are not specialists of *D. suzukii* (van Alphen and Janssen 1982; Carton et al. 1986; Mitsui et al. 2007; Ideo et al. 2008; Novković et al. 2011). Therefore, the *suzukii*-associated type of *G. xanthopoda* would be the best agent for biological control of *D. suzukii*.

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Table 1 Individual numbers of parasitoids (*G*: *Ganaspis* individuals, *Lj*: *Leptopilina japonica*, *A*spp: *Asobara* spp.) that emerged from *D. suzukii* pupae obtained from wild cherry fruits collected in Naganuma park, Tokyo, in June 2010 and 2011.

Collection Site	Position	No. of fruits	No. of pupae	No. of wasp individuals		
				G	*Lj*	*A*spp
2010						
A	On tree	319	367	26	-	-
A	On ground	737	1152	21	-	5
B	On ground	1772	1528	63	-	2
C	On ground	1064	891	57	2	-
2011						
	On ground	>1000	NC	112	10	-

NC: Not counted.

Table 2 Individual numbers of flies and parasitoids that emerged in parasitism experiments.

Host species	Cherry			Drosophila medium			s
	No. of replicates (vials)	Flies	Parasitoids	No. of replicates (vials)	Flies	Parasitoids	
D. suzukii	17	408	129	4	174	0	
D. lutescens	5	148	0	-			
D. rufa	2	121	0	-			
D. auraria	2	318	0	-			
D. biauraria	1	16	0	-			
D. triauraria	2	196	0	-			

Figure legends

Fig. 1 Position of the 7 landmarks used in geometric morphometric analyses on the right forewing of a *Ganaspis* specimen

Fig. 2 Neighbor-joining trees for *Ganaspis* individuals that emerged from *D. suzukii* and *D. lutescens* collected from Tokyo and Sendai based on COI (573 bp), ITS1 (334 bp) and ITS2 (357 bp) nucleotide sequences. Individuals are mentioned with the localities they have originated (Tokyo or Sendai), the host species (in parentheses: *D. suzukii* or *D. lutescens*), individual numbers, and Accession numbers (in parentheses). *Leptopilina japonica japonica* and *L. heterotoma* are used as outgroup in COI tree, and no outgroup was used in ITS1 and ITS2 trees. Bootstrap values are given above the branches supporting groups (values below 50% are omitted)

Fig. 3 Distribution of the first canonical variate scores based on morphometric analyses on forewing shape and antenna

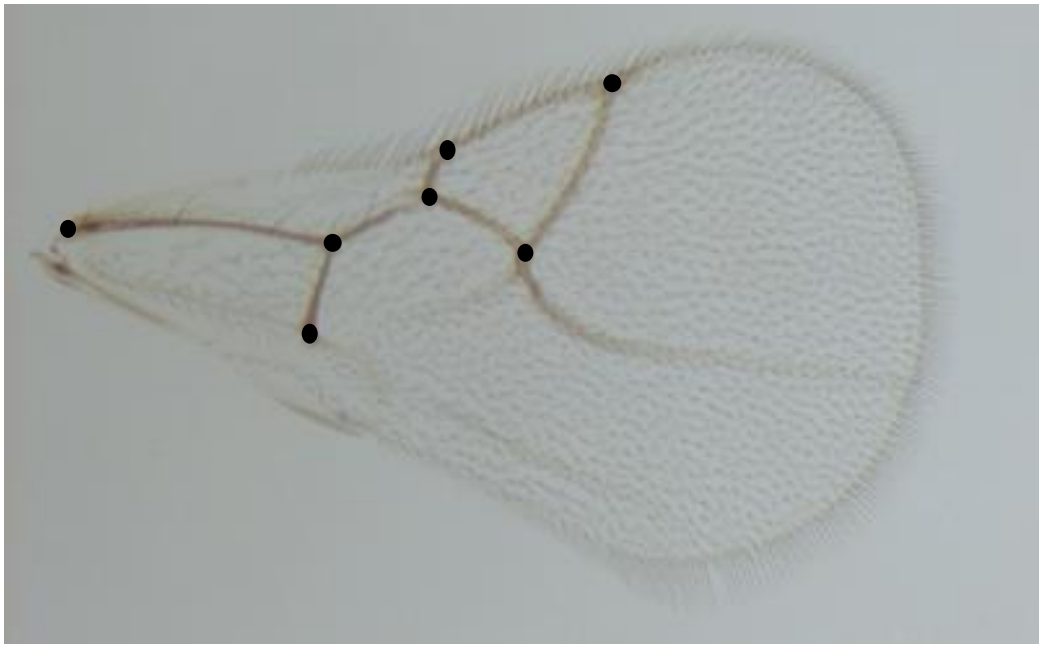
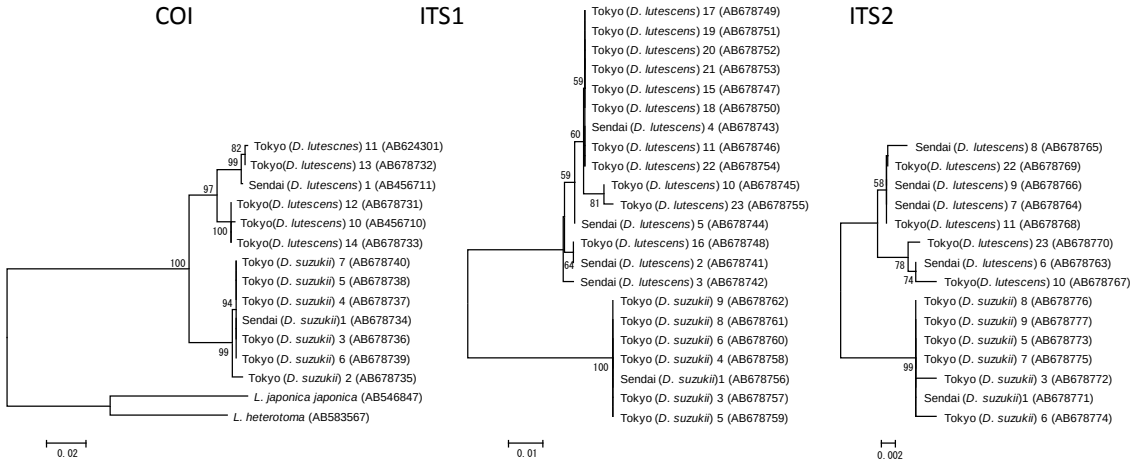


Fig. 1


COI
ITS1
ITS2
Tokyo (D. lutescnes) 11 (AB624301)
Tokyo(D. lutescens) 13 (AB678732)
Sendai (D. lutescens) 1 (AB456711)
Tokyo(D. lutescens) 12 (AB678731)
Tokyo(D. lutescens) 10 (AB456710)
Tokyo(D. lutescens) 14 (AB678733)
Tokyo (D. suzukii) 7 (AB678740)
Tokyo (D. suzukii) 5 (AB678738)
Tokyo (D. suzukii) 4 (AB678737)
Sendai (D. suzukii)1 (AB678734)
Tokyo (D. suzukii) 3 (AB678736)
Tokyo (D. suzukii) 6 (AB678739)
Tokyo (D. suzukii) 2 (AB678735)
L. japonica japonica (AB546847)
L. heterotoma (AB583567)
0. 02
Tokyo (D. lutescens) 17 (AB678749)
Tokyo (D. lutescens) 19 (AB678751)
Tokyo (D. lutescens) 20 (AB678752)
Tokyo (D. lutescens) 21 (AB678753)
Tokyo (D. lutescens) 15 (AB678747)
Tokyo (D. lutescens) 18 (AB678750)
Sendai (D. lutescens) 4 (AB678743)
Tokyo (D. lutescens) 11 (AB678746)
Tokyo (D. lutescens) 22 (AB678754)
Tokyo (D. lutescens) 10 (AB678745)
Tokyo (D. lutescens) 23 (AB678755)
Sendai (D. lutescens) 5 (AB678744)
Tokyo (D. lutescens) 16 (AB678748)
Sendai (D. lutescens) 2 (AB678741)
Sendai (D. lutescens) 3 (AB678742)
Tokyo (D. suzukii) 9 (AB678762)
Tokyo (D. suzukii) 8 (AB678761)
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Tokyo (D. suzukii) 6 (AB678774)
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Fig. 2

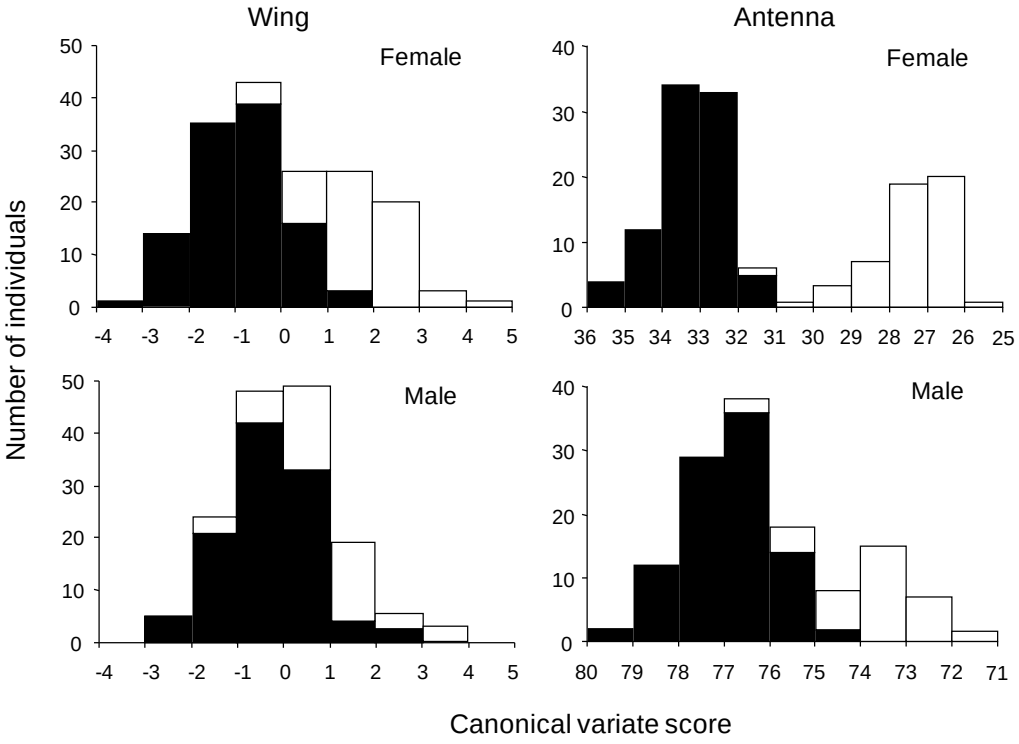
Wing
Antenna
Female
Female
Male
Male
Number of individuals
Canonical variate score

Fig. 3