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Genetic differences between rice and water-oat feeders in the rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae)

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Abstract
The rice stem borer, *Chilo suppressalis* (Walker) is an important lepidopteran pest of rice plants in Japan, and feeds on both rice (*Oryza sativa*) and water-oats (*Zizania latifolia*). We evaluated the difference in mating time of the two feeders under both laboratory and field conditions. The male moth of the water-oat feeders (WF) began to mate 7 h after the beginning of the scotophase, about 4 h later than the peak of mating of the rice feeders (RF). In accordance with this result, the body size of males trapped using synthetic sex pheromones differed between the two trap periods (sunset to 1:00 am vs. 1:00 am to sunrise) in the field, suggesting that the rice feeders were trapped earlier than the water-oat feeders. However, there was no unequivocal evidence showing limited gene flow between the two feeders at the level of allozyme polymorphisms. These results suggest that as the two feeders of *C. suppressalis* have recently diverged, the allozyme loci have not yet diverged to fixation.

Key words: *Chilo suppressalis*; mating time; reproductive isolation; allozymes

INTRODUCTION

The rice stem borer moth, *Chilo suppressalis* (Walker) is an important insect pest of rice plants in Japan (Kiritani, 1988), and feeds on both rice (*Oryza sativa*) and water-oats (*Zizania latifolia*). Many studies have tried to detect the difference between rice feeders and water-oat feeders phenotypically and behaviorally. Although some researchers agree that the body size and mass of adults and young collected from water-oats are larger than those from rice (e.g. Maki and Yamashita, 1956; Takasaki et al., 1969), earlier studies failed to detect any phenotypic and karyotypic differences between the two feeders (Kurihara, 1930; Marumo, 1930), showing that the young of both feeders has no host plant preference and reaches full growth with both host plants (Maki and Yamashita, 1956; Takano et al., 1959). Koike et al. (1981) showed that adult females of the two feeders do not exhibit any ovipositional preference of one host over the other, and a viable F_2 generation was obtained under laboratory conditions. These authors therefore concluded that there was no evidence of reproductive isolation between the two feeders. However, Konno and Tanaka (1996a) showed significant differences between the two feeders in insecticide susceptibility and activity of the aliesterase isozyme using moths collected in Okayama Prefecture. They also observed a clear difference in mating time between the two feeders under laboratory conditions: rice feeders mostly mated during the first half of the scotophase, while water-oat feeders mated during the late scotophase in moths from Okayama Prefecture (Konno and Tanaka, 1996b). These findings suggest that there could be a certain reproductive barrier between the two feeders. If this is true, the difference in mating time should be a possible mechanism for their reproductive isolation. Although there are several intriguing reports and/or considerations about reproductive isolation between the two feeders, no one has yet evaluated the magnitude of gene flow between them using genetic markers. If there is limited gene flow between the two feeders, (1) water-oats may not act as a natural refuge plant for rice feeders (see Bourguet et al., 2000a, b, for European corn borer *Ostrinia nubilalis*), and the removal of water-oats

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growing around rice fields may be meaningless to reduce the population density of the rice stem borer moth, (2) the two feeders might be midway in sympatric speciation, and could contribute to our deeper understanding of the mechanism of sympatric speciation (e.g., Feder et al., 1998; Drés and Mallet, 2002). Therefore, this study aimed to evaluate the level of population subdivision between the two feeders using allozyme markers. To confirm that the difference of mating time between the two feeders is not dependent on locality, we evaluated the difference in mating time of the two feeders under both laboratory and field conditions using moths collected in Gifu Prefecture, central Japan.

MATERIALS AND METHODS

Study population. Larvae of rice feeders (RF) and water-oat feeders (WF) were collected in Kaizu town, Gifu Prefecture. We collected RF larvae from the remaining stub of rice plants after harvesting in March 1997, and WF larvae from the dead stems of water-oats in creeks around paddy fields from December 1996 to March 1997. The two types of larvae were separately reared on rice seedlings under laboratory conditions (15L : 9D, 25±1°C) according to Sato (1964), and the resulting adult moths were used for the observation of mating time. We also collected overwintering larvae from November 1997 to February 1998 and in October 1998 and reared them as described above. The resulting adult moths were used for electrophoresis and the measurement of wing length. The negative effect of rice seedling supply on the growth of overwintering larvae seemed insubstantial, because most larvae were last instars, and eventually pupated after feeding on a small portion of seedlings under our rearing conditions.

Observation of mating time under laboratory conditions. Equal numbers of male and female adults (1 d post-emergence) of the two feeders (6–10 pairs) were released at least 1 h before the beginning of the scotophase in a cage covered with fine mesh (300 mm×300 mm×300 mm), as reported by Konno and Tanaka (1996b). Laboratory conditions were kept constant (15L : 9D, 25±1°C) for observation. We checked the number of mating pairs every 15 min, using a flashlight covered with red cellophane during the laboratory-induced scotophase. The observation was conducted five and four times for RF and WF, respectively.

Pheromone trap catch study. In Kaizu town, water-oats grow naturally along creeks around paddy fields. We placed pheromone traps 1.5 m above the ground along such creeks from the end of April to September 1998. We used a rubber septum containing synthetic pheromone (Shin-Etsu Chemical Co., Ltd., Japan) that was placed at the center of a sticky plate (240 mm×250 mm, Takeda Chemical Industries Co., Ltd., Japan) as a pheromone trap. Konno and Tanaka (1996b) and Samudra et al. (2002) reported that the mating time differed between the two feeders under laboratory conditions, and if this was the case in our study populations, the sizes and gene frequencies could be differentiated between the trap periods corresponding to different mating activities between the two feeders. Therefore, we collected trapped moths at both midnight and early morning: the traps were set before sunset, the trapped moths (hereafter, first trap samples) were gently collected alive at 1:00 am, a new sticky plate with the pheromone was left until the next morning, and the moths trapped on the new plate (hereafter, second trap samples) were collected in the early morning. Every collected moth was stored at −80°C. The number of first trap samples during the second (and third) flight season (16 June to 30 September 1998) was very low (n=3), possibly due to pesticide application in the rice fields; therefore, we excluded the data of both trap samples in the second flight season from our analyses.

Measurement of wing length. To avoid the reduction of sample sizes due to wing destruction when detaching samples from the sticky traps, we measured the length of seven parts of the fore and hind wings (Fig. 1) of each type of male moth scanned by a CCD camera (Olympus color camera cs220, Tokyo Electronic Industry Co., Ltd.) attached to a microscope connected to an Apple Macintosh computer and measured with NIH image 1.62 (public domain software).

Electrophoresis. We collected RF and WF larvae in the field from November 1997 to February 1998 and in October 1998. The larvae were reared using the method mentioned above and the resulting adult moths were stored at −80°C. The stored samples, including the first and second trap samples, were homogenized in 0.14 ml buffer (7 mM

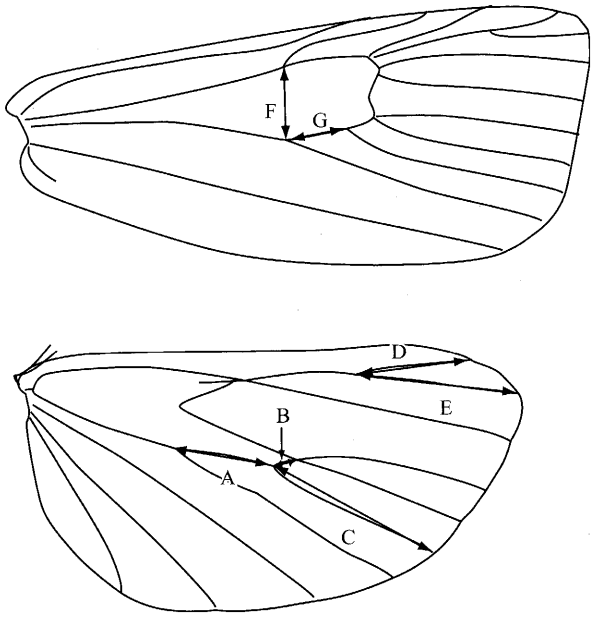

Fig. 1. Measured lengths of the fore and hind wings in male *Chilo suppressalis*. Wing images were captured by CCD camera and each length was measured using NIH image software.

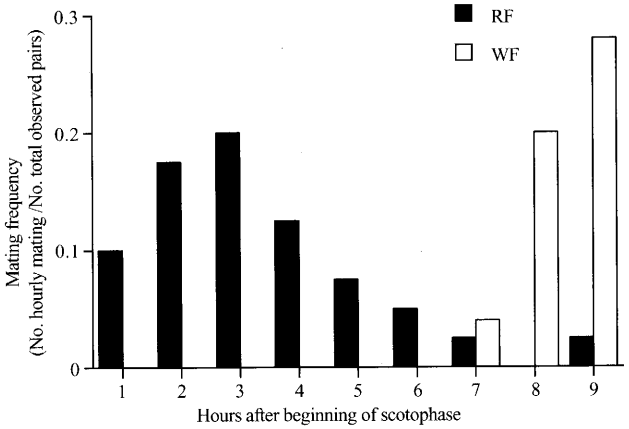

Fig. 2. Hourly changes in mating frequency of rice feeders (RF; $n=40$) and water-oat feeders (WF; $n=25$) in *C. suppressalis*. There was a significant difference in mating time between RF and WF (Mann-Whitney test, $U=10$, df=1, $p<0.01$).

Tris-HCl pH 7.0, 14% glycerine, 0.7% β-mercapto ethanol) and centrifuged at 18,000×g at 4°C for 15 min. The supernatants were collected and stored at −80°C until electrophoretic analysis. Gels with samples soaked in filter paper wicks were run with a standard horizontal electrophoretic system using starch gels (13% w/v) under 60 mA constant for 4 h for isocitrate dehydrogenase (IDH, E.C. number 1.1.1.42) and phosphoglucomutase (PGM, 5.4.2.2), and 140 V constant for 24 h for glucose-6-phosphate isomerase (GPI, 5.3.1.9), and stained according to the standard protocol of Murphy et al. (1996). We used the buffer system described by Varvio-Aho and Pamilo (1980).

Wright's fixation index (F) was calculated by the equation (Nei and Chesser, 1983):

$$\hat{F}=1-\hat{H}_o/\hat{H}_s,$$

where $\hat{H}_o$ is the unbiased estimate of the frequency of observed heterozygosity, and $\hat{H}_s$ is the unbiased estimate of the frequency of expected heterozygosity, which is calculated by the equation:

$$\hat{H}_s=\frac{N}{N-1}\left[1-\sum p_i^2-\frac{\hat{H}_o}{2N}\right],$$

where N is the number of sampling individuals and p_i is the frequency of the i-th allele at a given locus. The statistical deviation of the index from zero was tested using Chi-square test:

$$\chi^2=N(k-1)F^2,$$

where k is the number of alleles and df=$k(k-1)/2$.

We also estimated genotypic difference between the populations using Fisher's exact test (GENEPOP web version, Raymond and Rousset, 1995a, b).

RESULTS

Mating behavior

RF males began to mate after 30 min from the beginning of the scotophase. The mating frequency peaked 3 h after the beginning of the phase and then gradually decreased. On the other hand, WF males began to mate 7 h after the phase began and the frequency peaked 9 h after the beginning of the phase. There was a significant difference in mating time between RF and WF males (Fig. 2; Mann-Whitney test, $U=10$, df=1, $p<0.01$).

Wing length

The mean (±SD) length of seven wing parts of RF and WF males, and two trap samples (first and second) are shown in Fig. 3. The mean wing length of WF males was significantly larger than that of RF males for all parts. The second trap samples were significantly larger than the first trap samples

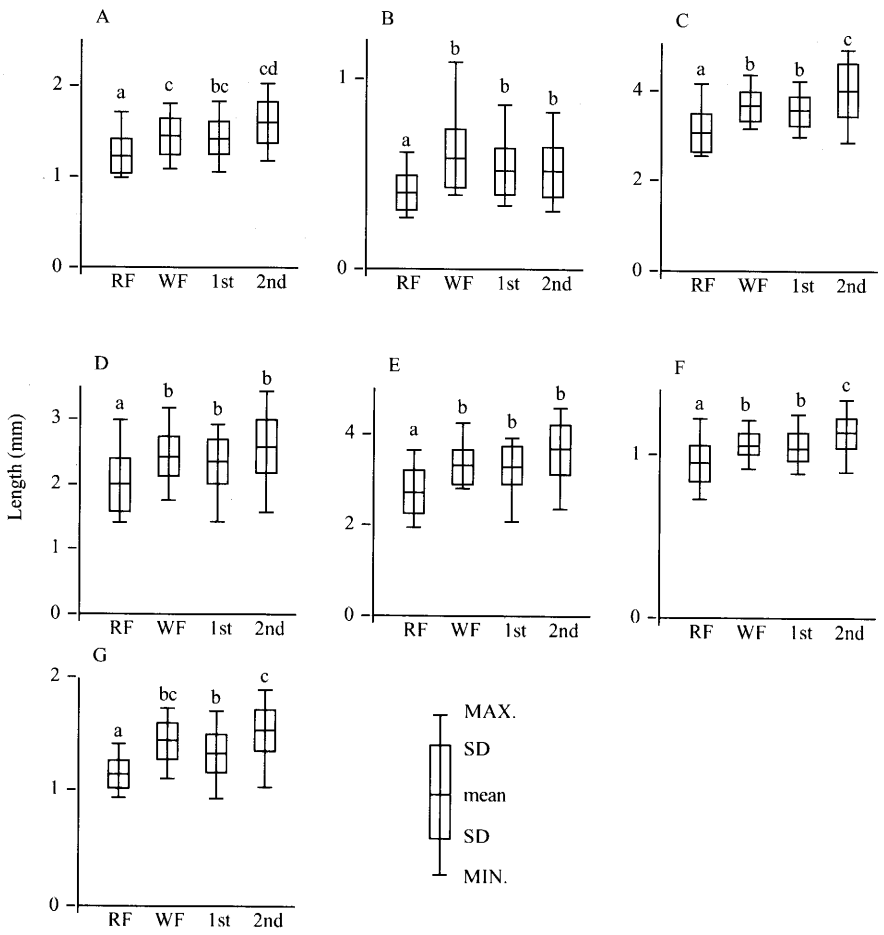

Fig. 3. Comparisons of the length of each wing part (mm) in male moths in the first flight season of *C. suppressalis*. See Fig. 1 for each measured part. RF indicates rice feeders (n=30). WF indicates water-oat feeders (n=35). The 1st means individuals trapped during the first period (n=37, sunset to 1:00 am). The 2nd means those trapped during the second period (n=32, 1:00 am to sunrise). Means followed by the same letter are not significantly different at 5% level by Scheffé's test.

for three wing parts (C, F and G). The means of three wing parts (A, D and E) of the second trap samples were also larger than those of first trap samples, but the difference was not statistically significant.

Genetic population structure

We successfully detected three, four and eight alleles at IDH, PGM and GPI, respectively. At other than the three loci, we could not detect any useful polymorphisms at ten allozyme loci (6-phosphogluconate dehydrogenase 1.1.1.44, malate dehydrogenase 1.1.1.37, aconitate hydratase 4.2.1.3, mannose-6-phosphate isomerase 5.3.1.8, creatine kinase 2.7.3.2, adenylate kinase 2.7.4.3, hexo kinase 2.7.1.1, phosphoglycerate kinase 2.7.2.3, glucose dehydrogenase 1.1.1.118 and malic enzyme 1.1.1.40). We did not check the sex of our collected samples upon homogenizing; therefore, the allele frequencies were calculated irrespective of their sex, other than the trap samples (only males). Allele frequencies for three polymorphic loci are shown in Table 1. We did not check the sex of our rearing samples; however, the difference of allele frequencies between sexes seemed minimal, because the allele frequencies between the rearing samples (female and male) and the trapped samples (male) in 1998 did not differ at each locus using Fisher's exact tests (using total samples, IDH; p=0.38, PGM; p=0.34, GPI; p=0.09). The allele frequencies were significantly different between the two feeders at IDH in both 1997 and 1998, and at PGM in 1998 (Fisher's exact tests, $p<0.05$), but not at GPI in the two years. The allele frequencies were significantly different between the two trap samples at PGM (Fisher's exact

Table 1. Allele frequencies for polymorphic loci in *C. suppressalis*

Sample locus			*n*	Allele								p[a]
				A	B	C	D	E	F	G	H	
1997[b]	IDH	RF	313	0.080	0.914	0.006	—[e]	—	—	—	—	
		WF	157	0.038	0.962	0.000	—	—	—	—	—	
		Total	470	0.066	0.930	0.004	—	—	—	—	—	0.001
	PGM	RF	313	0.040	0.887	0.073	0.000	—	—	—	—	
		WF	157	0.045	0.866	0.076	0.013	—	—	—	—	
		Total	470	0.041	0.880	0.074	0.004	—	—	—	—	0.057
	GPI	RF	312	0.002	0.002	0.712	0.229	0.051	0.000	0.002	0.003	
		WF	157	0.000	0.000	0.685	0.242	0.064	0.003	0.000	0.006	
		Total	469	0.001	0.001	0.703	0.233	0.055	0.001	0.001	0.004	0.742
1998[c]	IDH	RF	103	0.068	0.932	0.000	—	—	—	—	—	
		WF	30	0.000	1.000	0.000	—	—	—	—	—	
		Total	133	0.053	0.947	0.000	—	—	—	—	—	0.043
	PGM	RF	103	0.024	0.951	0.024	0.000	—	—	—	—	
		WF	30	0.133	0.783	0.067	0.017	—	—	—	—	
		Total	133	0.049	0.914	0.034	0.004	—	—	—	—	<0.001
	GPI	RF	103	0.000	0.000	0.710	0.232	0.024	0.000	0.000	0.034	
		WF	30	0.000	0.000	0.661	0.305	0.034	0.000	0.000	0.000	
		Total	133	0.000	0.000	0.699	0.248	0.026	0.000	0.000	0.026	0.389
PT[d]	IDH	1st	42	0.024	0.963	0.012	—	—	—	—	—	
		2nd	36	0.056	0.944	0.000	—	—	—	—	—	
		Total	78	0.038	0.955	0.006	—	—	—	—	—	0.420
	PGM	1st	42	0.024	0.976	0.000	—	—	—	—	—	
		2nd	36	0.014	0.889	0.097	—	—	—	—	—	
		Total	78	0.019	0.936	0.045	—	—	—	—	—	<0.001
	GPI	1st	42	0.000	0.000	0.738	0.190	0.060	0.000	0.000	0.012	
		2nd	36	0.000	0.000	0.736	0.194	0.056	0.000	0.014	0.000	
		Total	78	0.000	0.000	0.737	0.192	0.057	0.000	0.006	0.006	0.964

[a] Differences of allele frequencies were compared by Fisher's exact test.
[b] These samples were collected in the field from November 1997 to February 1998.
[c] These samples were collected in the field in October 1998.
[d] Samples were trapped using synthetic sex pheromones.
[e] Not detected.

test, $p<0.001$), but not at the other two loci. There was no consistent evidence showing differences in gene frequencies between the two feeders and between the two trap samples across the loci. Observed heterozygosities and calculated fixation indices are shown in Table 2. We detected significant excesses of homozygosity at GPI in 1997, but the remaining F values did not differ from zero.

DISCUSSION

Our studies revealed that mating time differed between the two feeders of *Chilo suppressalis* under laboratory conditions. In accordance with this result, the body sizes of the trapped male moths were also different between the two different trap periods during the night in the field. In contrast, our genetic studies using allozyme polymorphisms failed to detect unequivocal evidence of population subdivision between the two feeders.

The results of our observation of mating time differed between the two feeders, showing the same trend as reported by Konno and Tanaka (1996b), using a population in Okayama Prefecture, and Samudra et al. (2002), using populations in Saitama and Ibaraki Prefectures. Recently, Ueno et al. (2006) also reported the same trend using a population in Niigata Prefecture. This trend appears to be spatially and temporally meaningful, because the species as RF and WF populations for each of these studies were collected at different times and in different localities. Thus, the delayed

Table 2. Observed heterozygosity and fixation index (F). Deviations from Hardy-Weinberg expection were calculated using Chi-square test.

Sample	Locus	Population	n	H_o[a]	F
1997[b]	IDH	pooled[e]	470	0.140	−0.007 ns
	GPI	pooled	469	0.394	0.126 *
		RF	312	0.343	0.217 *
		WF	157	0.491	−0.047 ns
	PGM	pooled	470	0.217	−0.002 ns
1998[c]	IDH	pooled	133	0.104	−0.056 ns
	GPI	pooled	133	0.432	0.037 ns
	PGM	pooled	134	0.157	−0.026 ns
PT[d]	IDH	pooled	78	0.089	−0.035 ns
	GPI	pooled	78	0.384	0.082 ns
	PGM	pooled	78	0.102	0.164 ns

* $p<0.01$.
[a] Observed heterozygosity.
[b] These samples were collected in the field from November 1997 to February 1998.
[c] These samples were collected in the field in October 1998.
[d] Samples were trapped using synthetic sex pheromones.
[e] Pooled samples included both RF and WF in 1997 and 1998 samples and both 1st- and 2nd-half-period samples in PT.

mating time in WF compared to RF seems to be a common feature of the rice stem borer, *C. suppressalis* in Japan. On the other hand, the range of mating time of RF in this study was wider than in the previous study (Konno and Tanaka, 1996b). In particular, some mating by RF was also observed at 7–9 h after the beginning of the scotophase, overlapping the time of WF mating.

The mean wing length of WF males was significantly larger than RF males for all parts. This result is in a good agreement with previous studies (Maki and Yamashita, 1956; Takasaki et al., 1969; Tsuchida and Ichihashi, 1995; Ishida et al., 2000). Kanno (1984) reported that the male sexual response to females was almost synchronized with the mating time of *C. suppressalis* in the two flight seasons. Therefore, we expected that our trap samples before and after 1:00 am could appropriately collect either RF or WF according to the differences in their mating time. According to this expectation, the second trap samples were larger than the first trap samples in three wing parts. These results suggest that the second trap samples included more WF adults than the first trap samples, and that RF males were attracted to the pheromone until about midnight while the WF males were more attracted after midnight.

WF males were significantly smaller than the second trap samples in two wing parts (Fig. 2, C and F); however, if the second trap samples mainly include WF males, we could expect them to be as large as the second samples. This result might be attributable to our rearing method, with rice seedlings for overwintering larvae having a negative effect on the larvae, and the resulting male moths not reaching the large size of WF males in the field. On the other hand, the sizes of the first trap samples were significantly larger than those of RF. There are three likely explanations for this contradiction: First, the first trap samples could include some WF males; second, the first trap samples could include hybrids of RF and WF feeding on water-oats in the field. Samudra et al. (2002) reported that cross-mating between WF and RF occurred and the resulting hybrid produced viable hybrid generation under laboratory conditions. The peak mating time of the F_1 generation was midway between those of their RF and WF parents (Samudra et al., 2002), corresponding to the time of our trap-exchanges. If such an F_1 generation occurred in our study area, these moths could have been trapped in both our trap periods. Mating frequency between WF females and RF males was significantly higher than the reverse (RF females×WF males) under laboratory conditions (Samudra et al., 2002), suggesting that WF females are more likely to mate with RF males than the reverse. These considerations suggest that the two feeders differ in

mating time, but the difference is not strict and some hybrid populations could also occur in the field; third, the first trap sample could include some offspring derived from RF having fed on water-oats. As the adult females of the two feeders oviposited both hosts without particular host preferences (Koike et al., 1981; Konno, 1998), we could not rule out the possibility that some RF females oviposit on water-oats and the resulting offspring grows on them.

There was no consistent evidence showing differences in gene frequencies between the two feeders and the two trap samples across the loci (Table 1); however, the gene frequencies significantly differed between RF and WF at IDH across the two years and at PGM in one of the two years. Similarly, the gene frequencies significantly differed between the two trap periods at PGM loci. These results suggest that there could be population subdivision due to limited gene flow between the two feeders at IDH and/or PGM. In contrast, eight of the nine calculated fixation indices (F) of pooled data were not significantly different from zero (Table 2). The remaining index at the GPI locus in 1997 showed significant deviations from Hardy-Weinberg expectation. In general, the index is positive when population subdivision (Wahlund effect) occurs within a focal population (e.g. Nei, 1987). The calculated index might be elevated by the Wahlund effect due to host plant difference. If population subdivision due to host plant difference is the only plausible factor to elevate the index, then recalculating the index separately for each candidate sub-structure (host plant difference) should lower the index; however, the index for RF remained significantly positive when we calculated the indices separately for RF and WF at GPI in 1997. Although further analyses are needed, in particular for IDH and PGM loci using more samples of more populations, we can safely conclude that there was no unequivocal evidence showing limited gene flow between the two feeders.

In this study, we revealed a clear difference in mating time, which is a candidate factor for reproductive isolation between RF and WF under laboratory conditions as reported by two previous reports (Konno and Tanaka, 1996b; Samudra et al., 2002). Additionally, the different trap periods corresponded well to the body size differences of trapped individuals, suggesting that the two feeders also differed in their mating time in the field. These results suggest that there could be population subdivision due to limited gene flow by differences in mating time between the two feeders; however, our allozyme analyses failed to detect unequivocal evidence of limited gene flow between the two feeders. This result also contradicts a previous study of the differences of aliesterase activities between the two feeders (Konno and Tanaka, 1996a). The study of *Spodoptera frugiperda* could be of help to understand this contradiction. There is a difference in mating time between corn-feeding and rice-feeding strains, and the corn strain has been shown to mate exclusively in the first two-thirds of the night whereas the rice strain mates in the last third (Pashley et al., 1992). Several behavioral, physiological, and genetic (mitochondrial DNA (mtDNA) and esterase) differences between the strains suggest that they were reproductively isolated from each other (Pashley, 1986, 1989; Prowell, 1998). However, the lack of strain specificity in the majority of amplified fragment length polymorphism (AFLP) markers and most allozymes supports the hypothesis that fall armyworm strains are recently evolved (Prowell et al., 2004). In general, AFLP and allozyme markers seem selectively neutral, and the loci did not diverge to fixation and alleles were shared between the two strains due to the retention of ancestral polymorphism. On the other hand, the few loci that diverged may be linked to loci in the regions of the genome associated with fitness and/or mate choice in each habitat (Prowell et al., 2004). In *C. suppressalis*, aliesterase activity, thought to be associated with detoxicate organophosphates, differed markedly between the two feeders (Konno and Tanaka, 1996a), because they have different histories of artificial selection pressure: an aliesterase variant with higher detoxication ability has spread in the population feeding on rice, whereas no such variant has spread in populations feeding on water-oats. These results and considerations suggest that the contradiction may be explained by the independence of our allozyme loci from the loci of detoxication. When populations associated with different habitats hybridize, between-habitat variation at neutral markers will tend to be retained in genomic regions linked to the loci under selection, even though the rest of the genome may become homogenized (Charlesworth et al., 1997; Emelianov et al., 2004). These consid-

erations suggest that as the two feeders of *C. suppressalis* have recently diverged, the loci have not yet diverged to fixation and alleles are shared between the two feeders due to the retention of ancestral polymorphism other than the loci under selection. In the near future, our series of studies using other genetic markers (microsatellites and mtDNA) should contribute to our further understanding of the level of gene flow between sympatric host strains of *C. suppressalis*.

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