Genetic Analysis of Pyriproxyfen Resistance in the Housefly, *Musca domestica* L.¹

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A housefly (*Musca domestica* L.) strain highly resistant to pyriproxyfen, a juvenile hormone analog, was established by laboratory selection of houseflies collected from the 3rd Yumenoshima dumping-island in Tokyo Bay, Japan. The strain developed 4,900-fold resistance to pyriproxyfen in the 3rd instar larvae by the 17th generation from a level of only 3.8-fold before selection and thus was named YPPF strain. Genetic analysis of pyriproxyfen resistance in YPPF houseflies revealed that resistance to pyriproxyfen in this strain was incompletely dominant and multifactorial. There were at least 4 dominant factors responsible for the resistance, with the most important factor being located on the 2nd chromosome and other factors on the 1st, 3rd and 5th chromosomes. Furthermore, bioassay of synergism showed that the resistance ratio in white pupae of houseflies was depressed to 29-fold from 400-fold by a cytochrome P450 inhibitor, 2-propynyl 2,3,6-trichlorophenyl ether (PTPE). These results indicate that P450 monooxygenase plays an important role in housefly resistance to pyriproxyfen.

Key words: juvenile hormone analog, pyriproxyfen, resistance, housefly, linkage analysis

INTRODUCTION

Insect juvenile hormone analogs (JHAs) are expected to be good agents for pest management because of their specific toxicity against target insect pests and their low deleterious effects on non-target species (RETNAKARAN et al., 1985). Pyriproxyfen, 4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether, is a relatively new JHA that has remarkable effects on mosquitoes (KAMIMURA and ARAKAWA, 1991), houseflies (HATAKOSHI et al., 1987; KAWADA et al., 1987), cockroaches (KAWADA et al., 1989), whiteflies (ISHAAYA and HOROWITZ, 1995) and tsetse flies (HARGROVE and LANGLEY, 1993). On the other hand, it can be easily metabolized and excreted by rats and mice (MATSUNAGA et al., 1995; YOSHINO et al., 1995).

Many previous studies have shown that various insect species have already developed resistance to JHAs (CERF and GEORGHIOU, 1972; DYTE, 1972; SPARKS and HAMMOCK, 1983; WILSON and TURNER, 1992), and it is no exception for pyriproxyfen. KAWADA et al. (1987) reported that several housefly strains with varying levels of resistance to organophosphorus (OP) or pyrethroid insecticides had lower susceptibilities to pyriproxyfen than a susceptible strain. BULL and MEOLA (1994) also found that, compared to a susceptible strain of housefly, a strain with high resistance to methyl parathion was about 32-fold less sensitive to

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pyriproxyfen. In the present study, we established a housefly strain resistant to pyriproxyfen in the laboratory and studied the genetics of the resistance. We also discuss the possible involvement of P450 monooxygenases in resistance mechanisms.

MATERIALS AND METHODS

Chemicals. Pyriproxyfen, technical product, 96.6% AI and 0.5% water-soluble formulation were kindly provided by Sumitomo Chemical Co., Ltd., Japan. Two synergists, piperonyl butoxide (PB), 5-[[2-(2-butoxyethoxy)ethoxy]methyl]-6-propyl-1,3-benzodioxole and 2-propynyl 2,3,6-trichlorophenyl ether (PTPE) were used in this study. PB was purchased from Wako Chemicals while PTPE was kindly provided by Nihon Bayer Agrochem K.K., Japan.

Housefly strains. (1) SRS, a WHO standard reference susceptible strain. (2) YS, an organophosphate-resistant strain collected from the 3rd Yumenoshima dumping-island in 1987 (YASUTOMI and MIHARA, 1988). (3) *ac;ar;bwb;ye;snp* (aabys), a susceptible strain bearing the recessive visible mutant markers: *ali-curve*, *aristapeda*, *brown body*, *yellow eyes* and *snip wings* on chromosomes 1 to 5 respectively. (4) YPPF, a pyriproxyfen resistant strain established from the YS strain after successive selections with pyriproxyfen for 17 generations and then kept in the laboratory with maintenance concentrations of pyriproxyfen every ten generations.

Housefly selection. The selection was conducted by medium-mixing method. Pyriproxyfen of 0.5% water-soluble formulation was used. The medium was prepared by mixing 25 g of insect food and 25 g of bran (Oriental Yeast Co., Ltd., Japan), then 50 ml of pyriproxyfen distilled water solution was added and mixed thoroughly. One hundred 3rd instar larvae were put into the medium and maintained until their emergence at $25 \pm 1^\circ\text{C}$, RH 60%, 16L-8D. Two or three concentrations of pyriproxyfen were used in selection of every generation. Selections for each concentration were replicated at least three times. Mortality of houseflies was determined from the percentage of the emerged adults, and was corrected with ABBOTT's formula. Adults emerged from the treatment which resulted in about 50% housefly mortality (LC_{50}) were collected for the next selection. Distilled water was used as controls and to adjust pyriproxyfen concentrations. The same methods were used for the bioassay of susceptibility to pyriproxyfen in each housefly strain.

Degree of dominance. The degree of dominance (D) of resistance in hybrids between the susceptible and resistant flies were calculated by the method of STONE (1968):

$$D = (2X_2 - X_1 - X_3) / (X_1 - X_3)$$

where X_1 , X_2 and X_3 represent the logarithms of LC_{50} values of the resistant (YPPF), F_1 hybrid and susceptible (aabys) houseflies, respectively. Reciprocal crossings were conducted as follows:

$$\begin{aligned} \text{Cross 1. } & \text{YPPF } \text{♀} \times \text{aabys } \text{♂} \\ \text{Cross 2. } & \text{aabys } \text{♀} \times \text{YPPF } \text{♂} \end{aligned}$$

Linkage analysis. The crossing design for linkage group analysis for the dominant factor of pyriproxyfen resistance in the YPPF strain, was based on the F_1 male-backcross method (TSUKAMOTO, 1964) as follows:

$$\text{aabys } \text{♀} \times F_1 (\text{aabys } \text{♀} \times \text{YPPF } \text{♂}) \text{♂}$$

pyriproxyfen treatments of 0.1 and 1.0 $\mu\text{g/g}$ medium to 3rd instar larvae of the backcross progenies were designed for susceptibility discrimination, treatment with distilled water served as a control and was used to determine the survival rate of each phenotype by the following formula:

$$\text{Survival rate} = (\text{Adults emerged from treatment})/(\text{Adults emerged from control})$$

6,600 larvae were used for each treatment and control. The percentage survival of each phenotype was transformed to the arc-sine and submitted to factorial analysis according to TSUKAMOTO (1964) to determine the linkage group of the resistance factor.

Bioassay of synergism. Because the white pupal stage is the most sensitive to pyriproxyfen in houseflies (ZHANG and SHONO, unpublished data), white pupae were used to test the effect of synergists. White pupae were topically treated with PB (50 $\mu\text{g/pupa}$) or PTPE (1 $\mu\text{g/pupa}$ for SRS, 5 $\mu\text{g/pupa}$ for YPPF) ethanol solutions and then treated immediately with a 0.5 μl methanol solution of pyriproxyfen. Treatments with synergists only served for controls. Each dosage of pyriproxyfen was replicated three times with 20 white pupae in each replication. Mortalities were determined from the emerged adults and corrected by ABBOTT's formula, the data were then subjected to probit linear analysis.

RESULTS

Effect of selection

YS strain housefly, which showed only 3.8 times resistance to pyriproxyfen before selection, developed high resistance to this chemical after selection in the laboratory, resistance ratio at LC_{50} was 4,900 fold in generation 17 (Fig. 1 and Table 1). YS strain showed a gentle slope in the log concentration-probit mortality line, suggesting that this strain was not homogeneous in its resistance to pyriproxyfen. Successive selection with pyriproxyfen brought a rapid development to a higher level of resistance and made the population homogeneous for pyriproxyfen resistance, as it can be seen from slopes of the regression lines of YS-12 and YS-17 in Fig. 1. The slope for generation 17 increased to 4.5, almost the same as for SRS and aabys strains (4.2 and 3.9 respectively), but it was only 1.9 in the

Fig. 1. The development of pyriproxyfen resistance in the 3rd-Yumenoshima strain (YS) of housefly under laboratory selection for 17 generations. SRS and aabys are susceptible strains. Selection and bioassays were conducted by mixing pyriproxyfen solution with medium of 3rd instar larvae. Mortality of houseflies was determined from the percentage of the emerged adults and was corrected with ABBOTT's formula.

Table 1. Larval susceptibilities of housefly strains to pyriproxyfen

Strains	LC ₅₀ ^a	Slope	RR ^b
YS-17	78	4.5	4,900
YS	0.060	1.9	3.8
aabys	0.021	3.9	1.3
SRS	0.016	4.2	1.0

^a Expressed as $\mu\text{g/g}$ medium.^b RR, resistance ratio, based on a value of 1.0 for SRS strain. Fig. 2. Resistance levels to pyriproxyfen in the resistant YPPF strain, susceptible aabys strain and their F₁ progenies. Bioassay conditions were the same as described in Fig. 1.Table 2. Toxicity of pyriproxyfen to parental houseflies (YPPF and aabys) and their F₁ progenies

Strains	LC ₅₀ ^a	RR ^b	<i>D</i> value ^c
YPPF	51	2,400	
F ₁ (YPPF ♀ × aabys ♂)	2.8	130	0.26
F ₁ (aabys ♀ × YPPF ♂)	2.7	130	0.25
aabys	0.021	1.0	

^a Expressed as $\mu\text{g/g}$ medium.^b RR, resistance ratio, based on a value of 1.0 for aabys strain.^c *D* value is the degree of dominance as indicated in the text.

original YS strain. A strain highly resistant to pyriproxyfen established after successive selection for 17 generations was named YPPF (Yumenoshima, pyriproxyfen resistant) and kept in the laboratory with pyriproxyfen concentrations of 50 $\mu\text{g/g}$ medium for every ten generations. This strain was used through out the study.

Susceptibility levels to pyriproxyfen

Results of bioassay for susceptibility to pyriproxyfen in parent YPPF and aabys strains, and their F₁ hybrids are shown in Table 2 and Fig. 2. The LC₅₀ of YPPF decreased from 78 to 51 $\mu\text{g/g}$ medium because of several generations of non-selection with pyriproxyfen. Resistance ratio of YPPF based on a susceptible aabys strain was 2,400 fold. F₁ hybrid flies

showed incomplete dominance in pyriproxyfen resistance. Dominance values for the F_1 hybrid of Cross 1 and Cross 2 were 0.26 and 0.25 respectively. Therefore, only the dominant effects of the resistance factors were investigated in the linkage group analysis by the F_1 male backcross method. The susceptibilities to pyriproxyfen of F_1 hybrids between susceptible marker males and YPPF females (Cross 1) and susceptible marker females and YPPF males (Cross 2) were almost the same (2.8 and 2.7 $\mu\text{g/g}$ medium, respectively), indicating the absence of cytoplasmic genetic factors or maternal influence on the resistance.

Linkage group analysis for dominant resistance factor

Survival rates of each phenotype of the backcross are shown in Table 3. More than half of the flies possessing *ar* character, which is located on the 2nd chromosome, were killed by 0.1 $\mu\text{g/g}$ medium pyriproxyfen and most of them were killed by 1.0 $\mu\text{g/g}$ medium pyriproxyfen, while survival rates for non-*ar* flies were high at both concentrations. The results clearly indicate that the dominant resistance factor exists on the 2nd chromosome. Survival rates transformed to arc-sine were examined by factorial analysis to confirm the effect of each chromosomal factor and the interaction among these chromosomes in pyriproxyfen resistance. Results of factorial analysis are summarized in Table 4. Chromosome 2 contributed the largest part of the resistance (at 0.1% level), and chromosomes 1 and 3 (at 1% level) also play significant roles, weak but significant effects were also observed in chromosome 5 (at 5% level). No interaction between chromosomes was found.

Synergism of pyriproxyfen in housefly resistance

Not only YPPF larvae had resistance to pyriproxyfen (Tables 1, 2), but also YPPF white pupae showed resistance to this chemical (Table 5), although selection was only done on larvae of houseflies by medium mixing method. Topical application of PTPE showed synergism to pyriproxyfen in YPPF houseflies although another P450 inhibitor PB had no synergistic effects (Table 5). LD_{50} of YPPF white pupae decreased from 27 ng/pupa to 1.3 ng/pupa when pretreated with PTPE; on the other hand, LD_{50} of SRS white pupae decreased from 0.067 ng/pupa to 0.045 ng/pupa. Thus the resistance ratio decreased from 400-fold to 29-fold.

DISCUSSION

Results of linkage group analysis indicate that resistance to pyriproxyfen in the YPPF strain of the housefly is multifactorial and factors on four chromosomes are responsible for resistance in this strain, with the most important factor being located on the 2nd chromosome and other factors on the 1st, 3rd and 5th chromosomes. Resistance related to P450 monooxygenase (MFO) has been associated with chromosomes 2 and 5 in the housefly (TSUKAMOTO, 1983). Recent studies showed that the regulatory gene for P450 in organophosphate-resistant "Rutgers" houseflies was located on the 2nd chromosome (CARIÑO et al., 1994), while the structural gene was on the 5th chromosome (COHEN et al., 1994). Our results showed that a P450 inhibitor PTPE decreased housefly resistance to a 29-fold level (Table 5), thus implicating increased oxidase activity as a resistance mechanism. The resistant gene(s) concerned with P450 monooxygenase is thought to be located on chromosomes 2 or 5 or both in the YPPF strain. SCOTT et al. (1984) also found that housefly resistance to permethrin in Learn strain (LPR strain) was multifactorial and related to factors on chromosomes 1, 2 and 3. Their further studies indicated that resistance related to P450 in LPR

Table 3. Relation between pyriproxyfen resistance and phenotypic expression of visible mutant markers in backcross progeny from the cross (aabys ♀ × F₁ (aabys ♀ YPPF ♂) ♂)^a

Phenotype (1;2;3;4;5)	No. in control	0.1 $\mu\text{g/g}$ medium			1.0 $\mu\text{g/g}$ medium			Mean θ
		No.	Survival rate		N	Survival rate		
			%	θ		%	θ	
+++;++++	250	309	123.60	90.00	210	84.00	66.42	78.21
a;++++	255	234	91.76	73.32	55	21.57	27.67	50.50
++a;+++	291	141	48.45	44.11	3	1.03	5.83	24.97
a;a;+++	188	67	35.64	36.65	1	0.53	4.18	20.42
+++;b;++	248	221	89.11	70.73	59	23.79	29.19	49.96
a;+;b;++	229	221	96.51	79.23	8	3.49	10.77	45.00
++a;b;++	214	37	17.29	24.57	0	0.00	0.00	12.29
a;a;b;++	162	5	3.09	10.12	0	0.00	0.00	5.06
+++;+;y;+	261	252	96.55	79.30	166	63.60	52.89	66.10
a;++++;y;+	252	202	80.16	63.55	36	14.29	22.21	42.88
++a;+;y;+	209	87	41.63	40.18	3	1.44	6.88	23.53
a;a;+;y;+	175	28	16.00	23.58	0	0.00	0.00	11.79
+++;b;y;+	207	205	99.03	84.36	48	23.19	28.79	56.57
a;+;b;y;+	174	162	93.10	74.77	4	2.30	8.72	41.75
++a;b;y;+	197	9	4.57	12.34	0	0.00	0.00	6.17
a;a;b;y;+	164	4	2.44	8.98	0	0.00	0.00	4.49
+++;+++;s	183	185	101.09	90.00	68	37.16	37.56	63.78
a;++++;s	163	153	93.87	75.66	10	6.13	14.34	45.00
++a;+++;s	176	27	15.34	23.06	0	0.00	0.00	11.53
a;a;+++;s	136	8	5.88	14.04	0	0.00	0.00	7.02
+++;b;+;s	157	151	96.18	78.73	8	5.10	13.05	45.89
a;+;b;+;s	141	125	88.65	70.31	2	1.42	6.84	38.58
++a;b;+;s	158	9	5.70	13.81	0	0.00	0.00	6.90
a;a;b;+;s	108	0	0.00	0.00	0	0.00	0.00	0.00
+++;+;y;s	176	175	99.43	85.68	50	28.41	32.21	58.94
a;++++;y;s	188	162	86.17	68.17	9	4.79	12.64	40.40
++a;+;y;s	162	11	6.79	15.10	0	0.00	0.00	7.55
a;a;+;y;s	132	5	3.79	11.22	0	0.00	0.00	5.61
+++;b;y;s	184	145	78.80	62.59	3	1.63	7.34	34.96
a;+;b;y;s	168	71	42.26	40.55	2	1.19	6.26	23.41
++a;b;y;s	173	1	0.58	4.36	0	0.00	0.00	2.18
a;a;b;y;s	99	0	0.00	0.00	0	0.00	0.00	0.00
Total	5,980	3,412		1,469.08	745		393.79	913.43

^a Every 6,600 larvae were used for each treatment and control.

houseflies was given by genes on the 1st and 2nd chromosomes, the structural gene for P450 in LPR was located on the 1st chromosome and the regulatory gene for P450 was supposedly located on the 2nd chromosome (LIU and SCOTT, 1995, 1996). This was the first report which showed that housefly resistance caused by P450 was linked to the 1st chromosome. It is still not known whether the resistant gene on chromosome 1 in the YPPF strain is also related to P450. Additionally, the result also showed that resistance to pyriproxyfen in YPPF housefly was related to the 3rd chromosome (Table 4). KOENER et al. (1993) showed that

Table 4. Factorial analysis of chromosomal effects on pyriproxyfen resistance using *ac;ar;bw;ye;snp 1;2;3;4;5*) multichromosomal mutant strain

Chromosome	Effect	Degree of freedom	Mean square	F value
1	167.64	1	1,756	7.66**
2	632.41	1	24,997	109.01***
3	185.02	1	2,140	9.33**
4	78.76	1	388	1.69
5	147.93	1	1,368	5.96*
1-2	86.17	1	464	2.02
1-3	54.35	1	185	0.81
1-4	-3.72	1	1	0.00
1-5	24.20	1	37	0.16
2-3	34.36	1	74	0.32
2-4	25.05	1	39	0.17
2-5	12.09	1	9	0.04
3-4	10.48	1	7	0.03
3-5	9.18	1	5	0.02
4-5	-12.51	1	10	0.04
1-2-3	44.84	1	126	0.55
1-2-4	-15.03	1	14	0.06
1-2-5	4.87	1	1	0.01
1-3-4	3.95	1	1	0.00
1-3-5	22.71	1	32	0.14
1-4-5	-10.29	1	7	0.03
2-3-4	2.38	1	0	0.00
2-3-5	-50.97	1	162	0.71
2-4-5	-25.79	1	42	0.18
3-4-5	42.48	1	113	0.49
1-2-3-4	33.73	1	71	0.31
1-2-3-5	2.67	1	0	0.00
1-2-4-5	7.58	1	4	0.02
1-3-4-5	-0.71	1	0	0.00
2-3-4-5	37.03	1	86	0.37
1-2-3-4-5	20.45	1	26	0.11
Error		31	229	

* Significant at 5% level.

** Significant at 1% level.

*** Significant at 0.1% level.

Table 5. Toxicity of pyriproxyfen with synergists to white pupae of susceptible SRS and resistant YPPF houseflies

Treatment	LD ₅₀ (ng/pupa)		RR ^a
	SRS	YPPF	
Pyriproxyfen alone	0.067	27	400
Pyriproxyfen + PB	0.055	19	350
Pyriproxyfen + PTPE	0.045	1.3	29

^a Resistance ratio = LD₅₀ YPPF/LD₅₀ SRS.

NADPH cytochrome P450 reductase gene of "Rutgers" houseflies was localized on chromosome 3. Whether P450 reductase activity has relationship with insecticide resistance of housefly is still unknown.

This study also indicated that one or more resistance mechanism(s) other than increased oxidase activity in YPPF housefly are presumed to be responsible for the 29-fold resistance remaining after synergism by PTPE. A possible resistance mechanism should be the decreased penetration of insecticides through the insect cuticle. And it is well known that decreased penetration is related to chromosome 3 in adult flies (TSUKAMOTO, 1983). BULL and MEOLA (1994) found that there were no differences between the 3rd instar larvae of a susceptible strain and an organophosphate-resistant strain of housefly in the cuticular penetration of pyriproxyfen, but this does not mean that resistance of pyriproxyfen in the YPPF housefly is not caused by decreased cuticular penetration, because the YPPF strain was established from the 3rd Yumenoshima housefly, which has a very large pool of various resistant genes and thus shows multi-resistant to almost all kinds of insecticides examined (SHONO, 1983). Another possible resistance mechanism should be related with juvenile hormone binding protein (JHBP) because JHBP may also modulate JH or JHA degradation at critical developmental stages (HAMMOCK, 1985; SHEMSHEDINI and WILSON, 1990). Consequently, resistance due to JHBP in YPPF strain may not be ignored.

PB and 2-propynyl ethers are well known as P450 inhibitors (CASIDA, 1970; FELLIG et al., 1970). Both PB and PTPE were effective on larvae of *Culex quinquefasciatus* SAY when used with permethrin (KASAI and SHONO, unpublished data), but PB had no synergistic effect with pyriproxyfen in YPPF larvae, as this study showed. Similar results showed that PTPE was effective with permethrin in resistant *Heliothis virescens* while PB had no synergistic activity (BROWN et al., 1996). These results may be due to the degradation of PB by insect enzymes (CASIDA, 1970). *In vitro* studies of pyriproxyfen metabolism with synergists in housefly microsomes are now in progress.

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