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Studies on the Phylogenetical Relationship in the *Henosepilachna vigintioctomaculata* Complex Based on Variation of Isozymes (Coleoptera : Coccinellidae)

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The *Henosepilachna vigintioctomaculata* complex is complicated. Besides *H. vigintioctomaculata* (=Hv) and *H. pustulosa*(=Hp), other questionable forms, the Western Tokyo form (=Ht) and Towada form epilachnas(=Hc), are found in Japan. Variation in the blood acid phosphatase and esterase isozymes of this group was examined using the disc electrophoresis technique on acrylamide gel. Judging from the number of isozyme bands per individual in each population, the Hv-complex was in the sequence of Hv<Hp<Ht≒Hc. The phylogenetical arrangement of this group based on acid phosphatase isozyme was shown. The Hv-complex was divided into two main groups, one being Hv and the other Hp, Ht and Hc. Of the latter three, Ht and Hc were found to bear a close relationship.

INTRODUCTION

The evolutionary relationship in the *Henosepilachna vigintioctomaculata* complex is complicated. Two major groups, *H. vigintioctomaculata* (MOTSCHULSY) and *H. pustulosa*(KÔNO) (abbreviated as Hv and Hp, respectively), are distributed in Japan. They are distinguished by external characters and food plants in natural conditions. The former feeds mainly on potato leaves and other solanaceous plants, while the latter mainly on thistle leaves (Compositae). In addition, two other questionable forms called the Western Tokyo form (Ht) and the Towada form epilachna (Hc) are known. The former has many points of similarity in external characters to Hp, though resembling Hv in food plants. The latter closely resembles Ht in external characters, but mainly feeds on *Caulophyllum robustum* MAXIM. (Berberidaceae) under field conditions. Therefore, the nomenclature of the Hv-complex is provisional. Some authors regarded Hv and Hp as two subspecies of the same species (e.g. YASUTOMI, 1966; SASAJI, 1971), some considered them to be mere intraspecific variations (e.g. LI and COOK, 1961), and some treated them as specifically distinctive (KATAKURA, 1974). Thus, the Hv-complex is a problem from the standpoint of speciation.

Some years ago, intra- and interspecific relationships and phylogenetic differentiation of these ladybirds were studied by biochemical methods, investigating protein and isozyme variability.

I have studied, mainly from the systematic point of view, isozymes in ladybirds using the disc electrophoresis technique on acrylamide gel. In the present paper, phylogenetical relationships of the Hv-complex are discussed by directly analysing their patterns on acrylamide gel.

MATERIALS AND METHODS

Population sampling. The samples came from 37 localities covering most of the geographic distribution of this group in Hokkaido and Honshu (Fig. 1). A list of populations is shown in Table 1. The materials used in this study were reared with potato leaves in the laboratory. About 15 hours before gathering blood from knee joints for regulating the sample, the ladybirds were starved in order to avoid complications due to host emzymes.

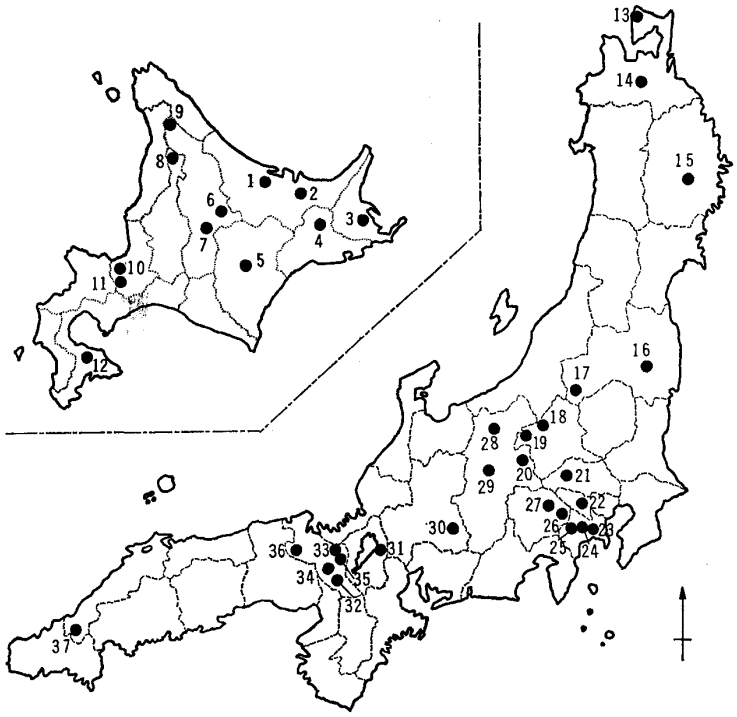

Fig. 1. Localities from which collected samples of the *Henosepilachna vigintioctomaculata* complex were studied, See the Table 1 for locality names.

Enzyme preparation. Ten mg of blood taken from one adult was mixed with 10 mg of 0.05 M potassium phosphate buffer, pH 6.8. The mixture was centrifuged at 3,000 rpm for 20 minutes, and then supernatant was mixed with the same volume of 1 M sucrose solution.

Electrophoretic technique. Electophoresis on acrylamide gel was performed as described by Aoki et al. (1966). Acrylamide gel columns were prepared in a glass tube 5 mm in diameter and 90 mm in length. The separating gel contains a 7.5% concentration of acrylamide and tris buffer, pH 8.9, and the large pore gel containing 3.75% acrylamide and tris buffer, pH 6.7, was overlaid on the separating gel. About 20 mg of a sample was placed on each gel column as a rule. Tris-glycine buffer, pH 8.6, was used for the run. Electrophoresis was carried out for 90 minutes with a constant current of 3 mA per column at 8±2°C, until the front marked by bromophenol blue migrated towards the anodic end.

Enzyme assays. The gel was carefully removed from the glass tube after completion of the electrophoresis and the gel was stained (Table 2). For observing the band,

Table 1. LOCALITIES AND NUMBERS OF ADULTS ASSAYED

Collection locality	Map number[a]	Date	No. of adults assayed	Host plant[b]
		Henosepilachna vigintioctomaculata		
Yasukuni	1	12 VIII 1975	24	P
Bihoro	2	13 VIII 1975	4	P
Odaitô	3	16 VIII 1975	6	P
Teshikaga	4	15 VIII 1975	10	P
Otofuke	5	17 VIII 1975	6	P
Otoineppu	9	18 VIII 1975	6	P
Misumai	10	19 VIII 1975	6	P
Ôma	13	21 VIII 1975	22	P
Tôno	15	23 IX 1975	8	P
Furudono	16	31 VIII 1975	6	P
Hinoemata	17	20 VI 1975	25	P
Shima	18	11 VI 1975	8	P
		23 VII 1975	8	P
Naganohara	19	11 VI 1975	6	P
Chichibu	21	5 V 1975	6	P
Akiyama	26	19 V 1975	12	P
Hajikano	27	4 V 1975	10	P
Togakushi	28	8 V 1975	4	P
Matsumoto	29	3 VI 1975	16	P
Nakatsugawa	30	13 VII 1975	6	E
Maibara	31	17 VI 1975	4	P
Ninose	32	26 VI 1975	6	P
Ashu	33	19 VII 1975	4	E
Seryo	34	19 VII 1975	8	P
Fukuchiyama	36	8 VI 1975	12	P
Tsuwano	37	14 VII 1975	6	E
		Henosepilachna pustulosa		
Sôunkyo	6	6 VIII 1975	30	T
Shirogane	7	7 VIII 1975	24	T
Shirakaba	8	19 VIII 1975	40	T
Misumai	10	19 VIII 1975	10	T
Ichinosawa	11	5 VIII 1975	8	T
Ônuma	12	21 VIII 1975	20	T
Ôma	13	21 VIII 1975	10	T
Tsuta	14	22 VIII 1975	22	T
Hinoemata	17	20 VI 1975	20	T, P
Shima	18	11 VI 1975	10	T, P
		23 VII 1975	16	T, P
Karuizawa	20	4 VI 1975	4	T
Togakushi	28	8 VII 1975	4	T
Ashu	33	28 VII 1975	8	T
Seryo	34	29 V 1975	8	T
		19 VII 1975	8	T, P
Tsuwano	37	14 VII 1975	10	T
		Western Tokyo form epilachna		
Hamura	19	3 V 1975	14	P
Zama	23	28 IV 1975	8	P

Table 1. (Continued)

Collection locality	Map number[a]	Date	No. of adults assayed	Host plant[b]
Atsugi	24	25 VIII 1975	24	P
Ochiai	25	25 VI 1975	16	P
		17 X 1975	14	E
Nakatsugawa	30	13 VII 1975	10	E, P
		Towada form epilachna		
Tsuta	14	22 VIII 1975	18	C
Togakushi	28	8 VII 1975	8	C

[a] Map numbers identify the locations in Fig. 1.
[b] P, potato; E, eggplant; T, thistle; C, *Caulophyllum robustum*.

Table 2. Staing Solution for the Enzyme

	Esterase[a]	Acid phosphatase[b]
Buffer	Potassium phosphate buffer, pH 6.8	Acetic acid buffer, pH 4.4
Substrate	α-Naphthyl acetate	α-Naphthyl phosphoric acid disodium salt
Dye coupler	Fast red RR salt	Fast red ITR salt

[a] Modified from Sasaji and Onishi (1973).
[b] Modified from Yoshitake et al. (1966).

the gel was put upon a lightbox, and bands were carefully recorded. To express the position of a certain band, relative mobility (abbreviated as Rm) was set on the basis of that of a BPB band used as the front marker.

$$\mathrm{Rm} = l_1 / l_2 \times 100$$

where l_1 was length between a given band and the origin, and l_2 was that of the BPB band. Three criteria were applied to characterize the isozyme patterns: a) the number of bands, b) the position of the bands and c) the density and width of bands.

RESULTS

Acid phosphatase activity of 493 samples from natural populations have been examined. The distinct bands were revealed on acrylamid gel, and these were numbered consecutively (Fig. 2). The band of Acph 3 at Rm 32–35 is broad and highly concentrated. The bands of Acph 1 and Acph 2, at Rm 47–49 and 51–53, respectively, are relatively broad but vary individually in concentration. The band of Acph 4 at Rm 24 is somewhat paler than Acph 3 but very sharp. Six slow-migrating bands, Acph 5 to Acph 10, are usually faint and low concentrated. The esterase activity of 261 samples were examined. More than ten bands appeared on the acrylamide gel. As studies on the substrate specificities of esterase are not included in this experiment, the discussion is limited to the number of bands per individual within a population.

The usefulness of electrophoretic results generally depends on the fulfillment of certain conditions: First, the patterns must be conservative enough to reflect the relationships within the Hv-complex; second, the patterns must be genetically determined and not modified by other factors; and third, the patterns which are obtained from various species or forms must be distinctive. The first condition is satisfied by

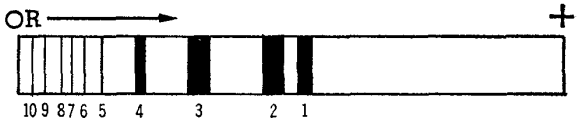

Fig. 2. Zymogram of the *Henosepilachna vigintioctomaculata* complex stained to show acid phosphatase.

a number of fractions with similar mobility in all groups of the Hv-complex. The second condition is met by the following observations. Ladybirds of the same species or forms collected from different places usually have the same pattern. The pattern of ladybirds kept in a refrigerator for two months was unchanged. There was no seasonal change of pattern in the same localities; Shima and Seryo(Hp), Shima(Hv) and Ochiai(Ht). Fulfillment of the third condition will be demonstrated in the following analysis.

Hv-Hp Mixed populations of Hv and Hp were found in potato fields in Hinoemata, Shima, Hirogawara, Seryo and Tsuwano. These fields have been cultivated together in a mountain village, and every stage of Hp was discovered from thistle growing in the vicinity of potato fields. The electrophoretic results of these two species are shown in Fig. 3. The member of Hp collected from a potato field possessed Acph 2 and its pattern agreed well with Hp feeding on thistle leaves.

Hv-Ht The geographical distribution of Ht was summarized in detail by YASUTOMI (1976). Sympatric populations of Hv and Ht were found in eggplant fields in Nakatsugawa, and each zymogram of acid phosphatase is shown in Fig. 4. The member of Hv has Acph 1, while Ht has Acph 2, in their populations. No intermediate pattern is recognized electrophoretically, and a distinction has been drawn between Hv and Ht by their external characters.

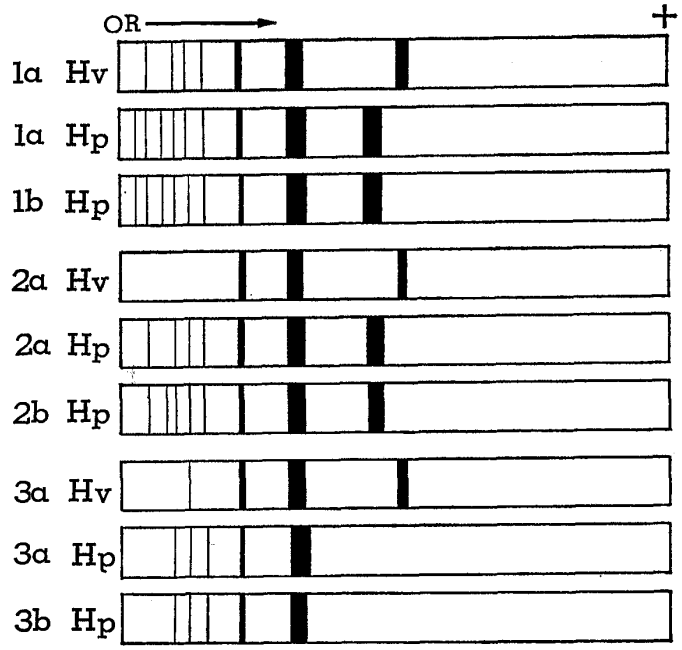

Fig. 3. Acid phosphatase pattern of the *Henosepilachna vigintioctomaculata* complex collected from Hinoemata(1), Shima(2), and Seryo(3).
a, feeding potato; b, feeding thistle. Hv, *H. vigintioctomaculata;* Hp, *H. pustulosa.*

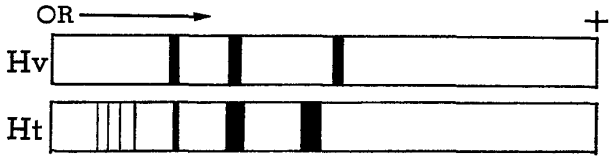

Fig. 4. Acid phosphatase pattern of the *Henosepilachna vigintioctomaculata* (Hv) and Western Tokyo form epilachna (Hc) collected from potato field of Nakatsugawa.

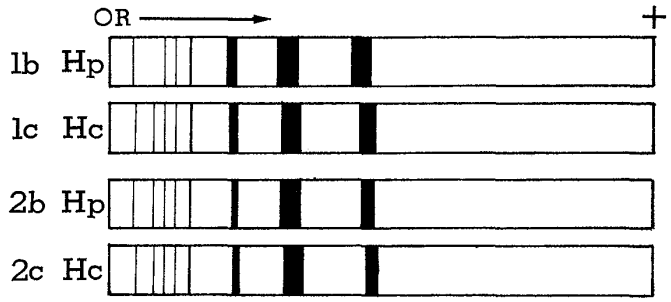

Fig. 5. Acid phosphatase pattern of *Henosepilachna pustulosa* (Hp) and Towada from epilachna (Hc) collected from Tsuta (1) and Togakushi (2).
b, feeding thistle; c, feeding *Caulophyllum robustum*.

Hp—Hc Neighboring populations of Hp and Hc were found in Tusta and Togakushi. Each zymogram of acid phosphatase is shown in Fig. 5. Both ladybirds have Acph 2 and no Acph 1. They were not distinguishable from each other in electrophoretic patterns. The member of Hc feeds on *Caulophyllum robustum* and *Panax japonicus* which constitute the flooring stratum of the forest community, while Hp feeds on thistles along a mountain stream. Thus, their habitats were isolated in different environments.

The frequency of occurrence of acid phosphatase isozyme bands among geographical populations is shown in Table 3. According to the occurrence of Acph 1 and Acph 2, Hv-complex can be divided into two main groups, one being Hv and the other Hp, Ht and Hc. The former possessed Acph 1, and the latter Acph 2. However, Hp of Shirakaba, Shirogane and Sôunkyo have no Acph 2. The band of Acph 1 occurs in high frequency in populations of central and western Honshu, while in northern Japan, it is found in low frequency in their populations. The band of Acph 2 of Ht appears to have reached fixation in all populations, except for that of Ochiai. In Ht it has been maintained in medium frequency. All assayed except for one specimen from Shima have Acph 3. The band of Acph 4 appears to have reached fixation (or near fixation) in Hp, Ht and Hc. In Hv, Acph 4 appears at a frequency of 100 percent in northern Japan, including Ôma, northern Tohoku District, but it gradually disappears in populations from Hinoemata, southern Tohoku District, and westwards to Honshu.

The number of isozyme bands per individual in each population is shown in Table 4. As to the number of acid phosphatase band, the relationship within the Hv-complex can be written as

$$Hv < Hp < Ht \fallingdotseq Hc.$$

Table 3. FREQUNCY OF ACID PHOSPHATASE ISOZYME BANDS (ACPH 1—4)

Collection locality[a]	Acph			
	4	3	2	1
	Henosepilachna vigintioctomaculata			
Yasukuni	100	100	0	25
Ôma	100	100	0	13
Tôno	100	100	0	50
Hinoemata	92	100	0	100
Hajikano	50	100	0	100
Matsutomo	67	100	0	75
Seryo	57	100	0	100
Fukuchiyama	75	100	0	67
	Henosepilachna pustulosa			
Sôunkyo	100	100	0	0
Shirogane	100	100	0	0
Shirakaba	100	100	0	0
Ônuma	100	100	86	0
Tsuta	92	100	50	0
Hinoemata	100	100	41	0
Shima	100	90	40	0
	100	100	43	0
Ashu	100	100	13	0
Seryo	100	100	0	0
Hirogawara	100	100	25	0
	Western Tokyo form epilachna			
Hamura	93	100	100	0
Atsugi	100	100	100	0
Ochiai	100	100	31	0
	100	100	36	0
Nakatsugawa	100	100	100	0
	Towada form epilachna			
Tsuta	100	100	25	0
Togakushi	100	100	71	0

[a] See Table 1.

Esterase also has a similar tendency with respect to the appearance of the acid phosphatase band.

DISCUSSION

The zymogram technique (electrophoretic separation of proteins combined with selective staining) permits a new and extensive examination of genetic differences between individuals, populations and species. HUBBY and THROCKMORTON (1965), who pioneered the use of electrophoretically demonstrable variation in systematics, discussed factors influencing the extent of genetic divergence at the protein level among species. Studies on genic similarity between *Drosophila* species have been reported (e.g. BERGER, 1970; RICHMOND, 1972). NEI (1972) has attempted to estimate the age of a species pair by relating genetic distance to time. SASAJI and ONISHI (1973)

Table 4. Number of Isozyme Band per Individual in Every Population[a]

Acid phosphatase		Esterase	
	Henosepilachna vigintioctomacula		
Yasukuni	3.8	Yasukuni	4.2
Ôma	3.7	Otofuke	4.4
Hinoemata	4.2	Ôma	4.3
Shima	2.4	Furudono	4.6
	2.7	Akiyama	4.7
Hajikano	2.5		
Matsumoto	2.4		
Seryo	2.9		
Fukuchiyama	3.0		
	Henosepilachna pustulosa		
Sôunkyo	4.6	Sôunkyo	4.8
Shirogane	5.2	Shirogane	6.1
Shirakaba	4.5	Shirakaba	6.0
Ônuma	5.1	Ônuma	5.6
Tsuta	4.0	Tsuta	5.2
Hinoemata	5.8	Shima	4.4
Shima	4.9		4.2
	4.6	Hirogawara	4.0
Ashu	3.8	Tsuwano	4.1
Seryo	3.6		
	3.9		
Hirogawara	4.8		
	Western Tokyo form epilachna		
Hamura	6.1	Zama	5.7
Atsugi	5.8	Atsugi	5.5
Ochiai	5.1	Ochiai	6.0
	5.2		6.1
Nakatsugawa	5.4		
	Towada form epilachna		
Tsuta	5.3	Tsuta	6.3
Togakushi	6.0		

[a] See Table 1.

reported some results of esterase isozymes from several species in Coccinellidae. They examined three adults and one larva of Hv collected in Fukui, and revealed five esterase bands.

Coccinellid beetles have the special characteristic (or reflex bleeding) that their blood is directly oozed from knee joints. In this experiment, the blood of ladybirds was used as the sample instead of the extract from the whole body.

Though a distinction has been drawn between the Honshu and Hokkaido forms of Hv by their external characters, there was no distinction between them at the isozyme level. Three bands, Acph 1, Acph 3 and Acph 4, were observed within the Shima population of Hv. Both Acph 1 and Acph 3 were fixed in this population; namely, the Shima population was composed of two types, one had Acph 1 and Acph 3, and the other Acph 1, Acph 3 and Acph 4. Similar phenomena was observed in the Hajikano, Chichibu, Nakatsugawa and Ninose populations. These populations

were located on the southern margin of the distribution range of Hv. Though Ht had more a fixed band than Hc in their populatins, there was no clear distinction between Ht and Hc. However, Acph 2 was fixed in all populations of Ht except for the Ochiai population. Generally, the original population has many changeable bands and shows high variability within a population. On the other hand, differentiated population shows low variability, and the number of fixed or lost bands increases in their population. YOSHITAKE (1968) examined genetic variability among geographical races of the silkworm, *Bombyx mori* L. for investigating the origin of their Japanese race. The Chinese univoltine race was the most polytypic of all races. He suggested that the Chinese univoltine race was the original one and that geographical races become differentiated from it.

According to the occurrence of Acph 1 and Acph 2, the Hv-complex was divided into two main groups, one being Hv and the other Hp, Ht and Hc (Table 3). Judging from the number of isozyme bands per individual in every population, the Hv-complex was in the sequence of Hv<Hp<Ht≒Hc (Table 4). The phylogenetical arrangement of the Hv-complex based on acid phosphatase isozyme is shown in Fig. 6. Resemblance between Ht and Hc was evident not only in external characters but also in isozyme patterns. The isozyme pattern of Hp was geographically very variable as compared with the other members of the Hv-complex. The Hp populations from Shirakaba, Shirogane and Sôunkyo, northern Hokkaido, have no Acph 2 in their populations. On the other hand, these populations showed substantially higher frequency of esterase bands than the other populations (Table 4). These ladybirds called the Typical form and the Sôunkyo form epilachnas had an isozyme pattern somewhat different from that in the other Hp.

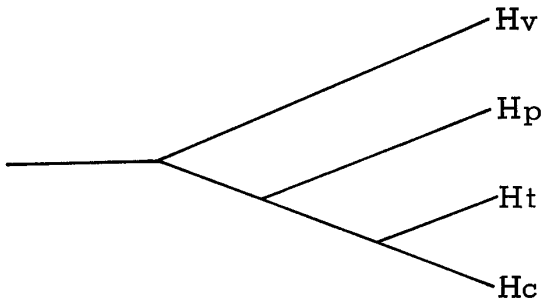

Fig. 6. Phylogenetical arrangement of the *Henosepilachna vigintioctomaculata* complex based on acid phosphatase isozyme. Hv, *H. vigintioctomaculata;* Hp, *H. pustulosa*; Ht, Western Tokyo form epilachna; Hc, Towada form epilachna.

KOYAMA (1962) compared the lesser tuberosity of the mandible within the Hv-complex, and pointed out that Ht was rather similar to Hp. KOYAMA and TAKIZAWA (1974) compared the marking patterns of larvae and pupae within the Hv-complex. Consequently, the pattern of Hc coincided closely with that of Ht, significantly differing from those of Hv and Hp. YASUTOMI (1976) pointed out a close relation between Ht and Hc on the basis of their preference for *Caulophyllum robustum.* These results do not contradict with the phylogenetical arrangement of the Hv-complex shown in Fig. 6.

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