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Karyotypic Differentiation in the Phytophagous Ladybird Beetles *Epilachna vigintioctomaculata* Complex and Its Possible Relevance to the Reproductive Isolation, with a Note on Supernumerary Y Chromosomes Found in *E. pustulosa*

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ABSTRACT—Chromosomes of phytophagous ladybird beetles belonging to the *Epilachna vigintioctomaculata* complex (Coccinellidae) were investigated in 18 populations. All the populations basically showed a diploid number of 20 with Xy_p sex chromosome associations in male meiosis. Detailed comparison of karyotypes using cluster and principal component analyses revealed a considerable divergence between the two groups of this species complex, the group A containing *E. vigintioctomaculata* and the group B including three other "species", *E. pustulosa, E. niponica,* and *E. yasutomii.* The divergence comes mainly from the addition of a large amount of heterochromatic segments on short arms of chromosomes Nos. 3 to 9 in karyotypes of the group B. This change in chromosomal structure confers the so-called diphasic state, which stands for a condition of chromosomes with one arm euchromatic and the other heterochromatic, in karyotypes of the group B species. The chromosome configurations observed suggested the late replication of heterochromatic arms in those diphasics. A new hypothetical model that accounts for the postmating reproductive isolation between the groups A and B is proposed on the basis of the karyotypic difference between the groups. Karyotypic differentiation within each group is also briefly mentioned. Significant differences were found among karyotypes of three populations of *E. vigintioctomaculata,* each of which represents one of three different geographic forms. Karyotypes of populations belonging to *E. yasutomii* deviated slightly from other species of the group B. Supernumerary Y chromosomes were found in males of some populations of *E. pustulosa* with considerable frequencies.

INTRODUCTION

The *vigintioctomaculata* complex of the genus *Epilachna* is a group of very closely related phytophagous ladybird beetles (Coleoptera: Coccinellidae) which are distributed mainly in the temperate regions of Japan. It consists of four "species", *E. vigintioctomaculata* Motschulsky, 1857, *pustulosa* Kôno, 1937, *niponica* Lewis, 1896, and *yasutomii* (Katakura, 1981) [12]. The species-complex has been greatly noticed over past two decades by researchers of diverse fields in evolutionary biology, especially by those who have particular interest in speciation process, due to their great variability in both external morphology and host plants [12, 16, 39].

Studies so far been made in detail with the aim to trace the possible causes of reproductive isolation among the members of the complex, are manifold, including phenology [11, 13], host plant preference [19], interspecific crossings [14, 15, 17–20, 24–25], and so on. Chromosomes of the complex have, however, been poorly known, although a few pioneer studies revealed that the chromosome composition of *vigintioctomaculata* and *pustulosa* was $2n=20$ with male heterogametic Xy_p-XX in sex determination [36–38, 43, 44]. The

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y in lower case stands for a minute Y chromosome and p in subscript in the Xy_p for a "parachute" which denotes the parachute-like configuration formed by a "canopy" X chromosome and a "load or parachutist" Y [33]. It seems that the failure to detect striking interspecific differences in karyotypes in those earlier studies using paraffin section or squash methods has discouraged further cytological studies on the *vigintioctomaculata* complex.

We have restudied chromosomes of 18 populations, which cover all the species of the complex, by the current air-drying method. As a result, we found that karyotypic divergence between *vigintioctomaculata* which belongs to the group A [12] and the other three species of the complex, referred to as the group B, is far more evident than previously thought. Furthermore, supernumerary Y chromosomes were found among some populations of *pustulosa* as an intrapopulation polymorphism. In this paper, we will present the data and discuss possible relevance of the karyotypic differentiation to postmating reproductive isolation exhibited by crossing between groups A and B.

MATERIALS AND METHODS

The specimens used for chromosome examination are listed in Table 1 (see also Fig. 1). The cytological data were obtained from air-dried preparations of testes or ovaries of adults just after eclosion, or supraoesophageal ganglia of the third and the fourth instar larvae. We dissected out materials from living individuals in hypotonic solution containing colchicine (mixture of 0.5 volume

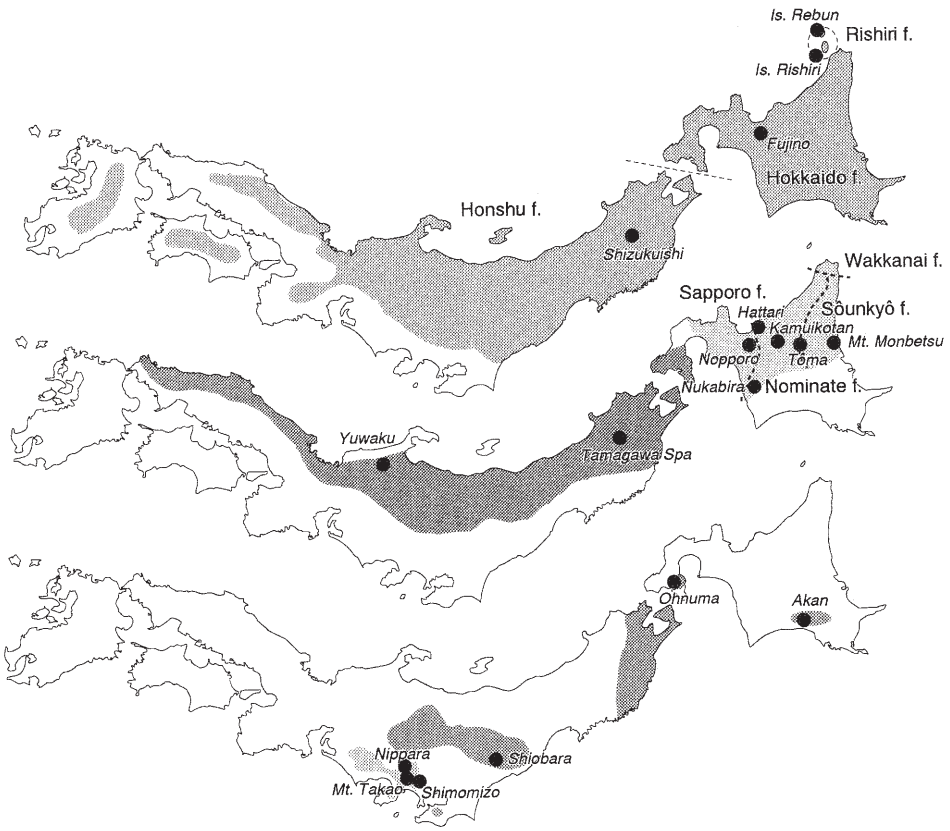

FIG. 1. Distribution of four species of the *Epilachna vigintioctomaculata* complex in Japan. Top: *E. vigintioctomaculata*. Middle: *E. niponica* (dark shade) and *E. pustulosa* (light shade). Bottom: *E. yasutomii* (dark shade) and Western Tokyo form (light shade). Solid circles denote populations used for the present chromosomal study.

TABLE 1. Chromosome numbers of the *Epilachna vigintioctomaculata* complex

Locality	Date	No. Indiv. examined[1)]	Chromosome number[2)]			Reference (PS= present study)
			Male		Female	
			2n	MI		
Epilachna vigintioctomaculata						
Honshu form						
Shizukuishi	28 June 1982	$2L_4$	20	—	20	PS
Hokkaido form						
?	—	—	20	9+Xy	—	[43, 44]
Hokkaido (5 sites)	—	—	—	$9+Xy_p$	—	[36]
Fujino, Sapporo	17 July to 10 Aug 1984	$7L_4$, 15♂4♀	20	$9+Xy_p$	20	PS
Rishiri form						
Motoji, Is. Rebun	12 July to 13 Aug 1984	16♂2♀, $7L_4$	20	$9+Xy_p$	20	PS
Nozuka, Is. Rishiri	8 July 1984	4♂	20	$9+Xy_p$	—	PS
Epilachna pustulosa						
Sapporo form						
?	—	—	20	?+Xy		[44]
Maruyama, Sapporo	—	—	—	$9+Xy_p$	—	[36]
Nopporo	28 Sept to 11 Oct 1982	3♂, 1♀ $11L_4$	20	$9+Xy_p$	20	PS
A population with an intermediate phenotype between Sapporo form and Nominate form						
Hattari	19 Aug 1984	24♂2♀, $2L_4$	20(19♂)	$9+Xy_p$	20	PS
			20+1B(5♂)	$9+Xyy_p$		
Nominate form						
Tokachi-Mitsumata	—	—	—	$9+Xy_p$	—	[36]
Mt. Taisetsu	—	—	—	$9+Xy_p$	—	[36]
Kamuikotan	28 June 1982	$4L_4$	20(1♂)	—	20	PS
			20+1B(1♂)			
Kamuikotan	11 June to 17 Aug 1984	22♂9♀	20(21♂)	$9+Xy_p$	20	PS
			20+1B(1♂)	$9+Xyy_p$		
Nukabira forest trail	4 Sept 1983	13♂2♀	20(13♂)	$9+Xy_p$	20	PS
A population with an intermediate phenotype between Nominate form and Sôunkyô form						
Tôma	4 Aug 1984	1♀	—	—	20	PS
Tôma	2 Sept 1985	6♂2♀	20(5♂)	$9+Xy_p$	20	PS
			20+1B(1♂)	$9+Xyy_p$		
Sôunkyô form						
Mt. Ohyama, Monbetsu	28 June to 28 July 1985	3♂1♀$2L_4$	20(2♂)	$9+Xy_p$	20	PS
			20+1B(1♂)	$9+Xyy_p$		

(continued)

TABLE 1. Continued

Locality	Date	No. Indiv. examined[1]	Chromosome number[2]			Reference (PS= present study)
			Male		Female	
			2n	MI		
Epilachna niponica						
Tamagawa Spa	30 June to 8 July 1983	4♂2♀9L_4	20	9+Xy_p	20	PS
Naeba	—	5♂	—	9+Xy_p(1♂) 9+Xyy_p(4♂)	—	[37]
Yuwaku, Kanazawa	17 to 18 July 1984	4♂2♀5L_4	20	9+Xy_p	20	PS
Yamanaka Pass, Tsuruga	—	2♂	20	9+Xy_p	—	[38]
Epilachna yasutomii						
Populations on *Caulophyllum robustum*						
Akan	26 Aug 1985	2♂	20	9+Xy_p	—	PS
Ohnuma	28 Sept to 5 Oct 1982	4L_4	20	—	20	PS
Asamushi	2 July 1982	1L_4	—	—	20	PS
Shiobara	4 July 1982	1L_4	20	—	—	PS
Western Tokyo form						
Shimomizo, on *Solanum tuberosum*	20 Oct to 10 Nov 1982	3♂1♀6L_4	20	9+Xy_p	20	PS
Mt. Takao, on *Chelidonium japonicum*	30 June to 4 July 1983	4♂7L_{3-4}	20	9+Xy_p	20	PS
Nippara, on *Scopolia japonica*	19 to 23 July 1983	3♂5♀	20	9+Xy_p	20	PS
Inter- or intraspecific hybrids obtained in laboratory[3]						
Ev ♀×*Ep* ♂	—	—	—	9+Xy_p	—	[36]
(*Ep* Sapporo f. ♀× *Ep* Nominate f. ♂)F_1 ♂×*Ep* Sapporo f. ♀	—	—	—	9+Xy_p	—	[36]
Ev Hokkaido f. ♀× *En* Ohnuma f. ♂	14 Sept 1982	1L_4	—	—	20	PS

[1] L_4: 4th instar larvae. L_{3-4}: 3rd and 4th instar larvae.
[2] Sex of larvae was determined by the presence or absence of small Y chromosome.
[3] *Ev*=*Epilachna vigintioctomaculata*, *Ep*=*E. pustulosa*, *En*=*E. niponica*.

of 0.1% colchicine solution and 9.5 volume of 1% sodium citrate) on a hollow slide. Then we macerated the materials for ca. 10 min. in the same solution before fixation with Carnoy's solution (3:1 volumes of absolute methanol and glacial acetic acid). The technique employed is the same as that for harvestman chromosomes described previously [40, 41]. In some cells, patterns similar to C-bands were observed, although these cells had received no special treatment.

We serially arranged mitotic metaphase chromosomes according to the descending order of length (Figs. 2, 3). We calculated percent ratios of length for each chromosome to the TCL, the total length of all haploid autosomes and an X chromosome. We draw haploid idiograms of each population

based on somatic metaphase plates from the clearest five, if available, chromosome configurations. We also calculated the arm ratio (r) of each chromosome by dividing the length of the long arm by that of the short arm, and classified chromosomes into the following five categories according to Levan et al. [21]: metacentric ($1.0 \leq r < 1.67$), submetacentric ($1.67 \leq r < 3.0$), subtelocentric ($3.0 \leq r < 7.0$), acrocentric ($7.0 \leq r < \infty$), and telocentric ($r = \infty$). The morphometric values calculated for 14 representative populations are shown in the Appendix.

To assess degrees of overall differentiation of karyotypes between any two populations, the method developed by Matsui [23] for the karyotype analysis of toads was applied with a slight modification. The procedure was as follows: (1) Choosing five chromosome plates for each population, we compared every homologous chromosome pair between the two karyotypes in two respects: relative length to TCL and the arm ratio (r), and performed Mann-Whitney *U*-tests ($P < 0.05$) for the statistical analysis. The Y chromosome was excluded from the comparison because sufficient data were unavailable in some populations due to the paucity of male specimens. (2) The result of each comparison then falls into any one of the following four categories: A - No differences in both length and arm ratio; B - Difference in length but not in ratio; C - difference in ratio but not in length; D - difference in both length and ratio. We gave the following ratings for these categories: A=2, B and C=1, and D=0. Thus, we can express the total similarity value of karyotypes from 0 (all the 9 autosome and one X chromosome pairs differ in both length and ratio) to 20 (no difference in both length and ratio for all 10 chromosome pairs). The similarity values thus obtained between populations are shown in upper right half of Table 2. The table also shows similarity coefficients which were calculated by dividing each similarity value by 20; thus the coefficients vary from 1.0 (perfect match) to 0 (perfect mismatch). On the basis of the similarity coefficients, a dendrogram using UPGMA [34] was depicted (Fig. 6).

To elucidate the factors responsible for the karyotypic differentiation in the *vigintioctomaculata* complex, the principal component analysis was also performed on the basis of 21 morphometrical characters of chromosomes for 14 populations (Table 3). Then the populations were projected on the first two axes (Fig. 7). All the statistical tests including the Mann-Whitney *U*-tests, cluster, and principal component analyses were implemented with the SYSTAT ver. 5.2 for the Macintosh personal computer [35].

RESULTS

Karyotypes

The chromosome number of the complex was invariably 2n=20 (Figs. 2, 3, Table 1) except for that of some males of *E. pustulosa* with a supernumerary Y chromosome (2n=21, Fig. 8B). All the chromosomes except for Y whose centromeric position is almost invisible due to its small size, were more or less diphasic, namely they were composed of a euchromatic arm and a heterochromatic one [33]. In diphasic chromosomes, sister chromatids of the heterochromatic arm coil together and the split between them is invisible when chromatids of the euchromatic arm have been already separated from each other, probably due to late replication and late condensation of the heterochromatic arm [33]. We inferred the diphasic nature of each chromosome primarily from the differential condensation between two arms. Some chromosomal plates which showed negative heteropycnosis between arms (see Figs. 2G, H, 3C) of almost all autosomes also support the inference. In the idiograms shown in Figs. 4 and 5, arms that inclined to make tight adherence between sister chromatids, hence supposed as heterochromatic arms, are crosshatched. The heterochromatic arms were almost always shorter than euchromatic ones on the opposite side.

However, those heterochromatic arms, virtually synonymous with short arms, do not consist exclusively of heterochromatin. For example, we supposed that distal halves of the heterochromatic arms in chromosomes No. 1 and X were euchromatic judging from configurations (see Figs. 2, 3) and banding patterns (cf. Figs. 2H, 3C) shown by those chromosomes. Furthermore, euchromatic

FIG. 2. Representative karyotypes of *Epilachna vigintioctomaculata* (A-D) and *E. pustulosa* (E-H). C and E from females (sex chromosome composition: XX); others from males (XY). A: Honshu form, Shizukuishi. B-C: Hokkaido f., Fujino. D, Rishiri f., Is. Rebun. E: Sapporo f., Nopporo. F: Nominate f., Kamuikotan. G: A population with an intermediate phenotype between Nominate f. and Sapporo f., Hattari. H: A population with an intermediate phenotype between Sôunkyô f. and Nominate f., Tôma. Scale =0.01 mm.

segments are also distributed in the distal ends of other chromosomes, namely autosomes of Nos. 2–9. Putative distribution of the heterochromatic and euchromatic segments in the karyotypes of the species-complex are diagrammatically represented in Fig. 10.

E. vigintioctomaculata (Figs. 2A-D, 4). Three populations representing three different geographic forms were surveyed. In Fujino population (the Hokkaido form), the autosomes consisted of a pair of large metacentrics (No. 1) and 8 pairs of submetacentrics (Nos. 2–9). In the other two populations, too, autosomes were similar except for 2 pairs of subtelocentrics (Nos. 3, 7) in Shizukuishi population (the Honshu form) and a pair of metacentrics (No. 2) in Is. Rebun population (the Rishiri form). The X chromosome, the largest in both Shizukuishi and Fujino populations, was submetacentric. The X in Is. Rebun population was

the third largest and differed in both relative length and arm ratio from those in the other two populations (Mann-Whitney *U*-tests, <0.05). Relative length of Y to X chromosome (Y/X ratio) in Rebun population (0.25 on average, n=5) also deviated from those in Fujino (0.18, n=5) and Shizukuishi (0.16, n=5) populations (Mann-Whitney *U*-tests, <0.05).

Epilachna pustulosa (Figs. 2E-H, 4). Six populations were surveyed. They include populations from Nopporo (the Sapporo form), Kamuikotan, Nukabira (the Nominate form), Monbetsu (the Sôunkyô form), Tôma (a population with an intermediate phenotype between the Nominate and the Sôunkyô forms), and Hattari (a population with an intermediate phenotype between the Nominate and the Sapporo forms). Of these, Monbetsu population, where no chromosomal spreads enough for detailed analysis were obtained, was excluded from the karyotype analysis. The autosomes consisted of 9 pairs of meta- or submetacentrics. The X chromosome was the second or the third largest subemeta- or metacentric chromosome. The ratio of total length of heterochromatic arms to that of euchromatic arms slightly but significantly varied among the five populations (Kruskal-Wallis test, $P<0.05$), although no difference was detected among three populations of those (Hattari, Kamuikotan, Nuka-

FIG. 3. Representative karyotypes of *Epilachna niponica* (A-B) and *E. yasutomii* (C-G). All, except for G, from males. A: Tamagawa Spa. B: Yuwaku. C: Akan. D: Shiobara. E: Western Tokyo form on *Solanum tuberosum,* Shimomizo. F, Western Tokyo form on *Chelidonium japonicum,* Mt. Takao. G: Western Tokyo form on *Scopolia japonica,* Nippara. Scale=0.01 mm.

bira) (Kruskal-Wallis test, $P=0.396$). Y/X ratios in five populations ranged from 0.20 to 0.32 and the difference among the populations was significant (Kruskal-Wallis test, $P<0.05$), though the interpopulation variation for two populations of the Nominate form was not evident (Kruskal-Wallis test, $P=0.053$).

E. niponica (Figs. 3A, B, 5). Two populations, one from Tamagawa Spa, Akita Pref., in northern Honshu and the other from Yuwaku, Kanazawa, central Honshu, were analyzed. Although the two populations are phenotypically considerably different, their karyotypes were very similar to each other in general configurations. Significant difference was found only in the relative length of a pair of chromosome No. 1 (U-test, <0.05).

E. yasutomii (Figs. 3C-G, 5). This species, feeding usually on *Caulophyllum robustum*, is taxonomically problematic because it shows much variability in host plant preference in spite of its rather conservative external morphology throughout its distributional range [12]. We treated here

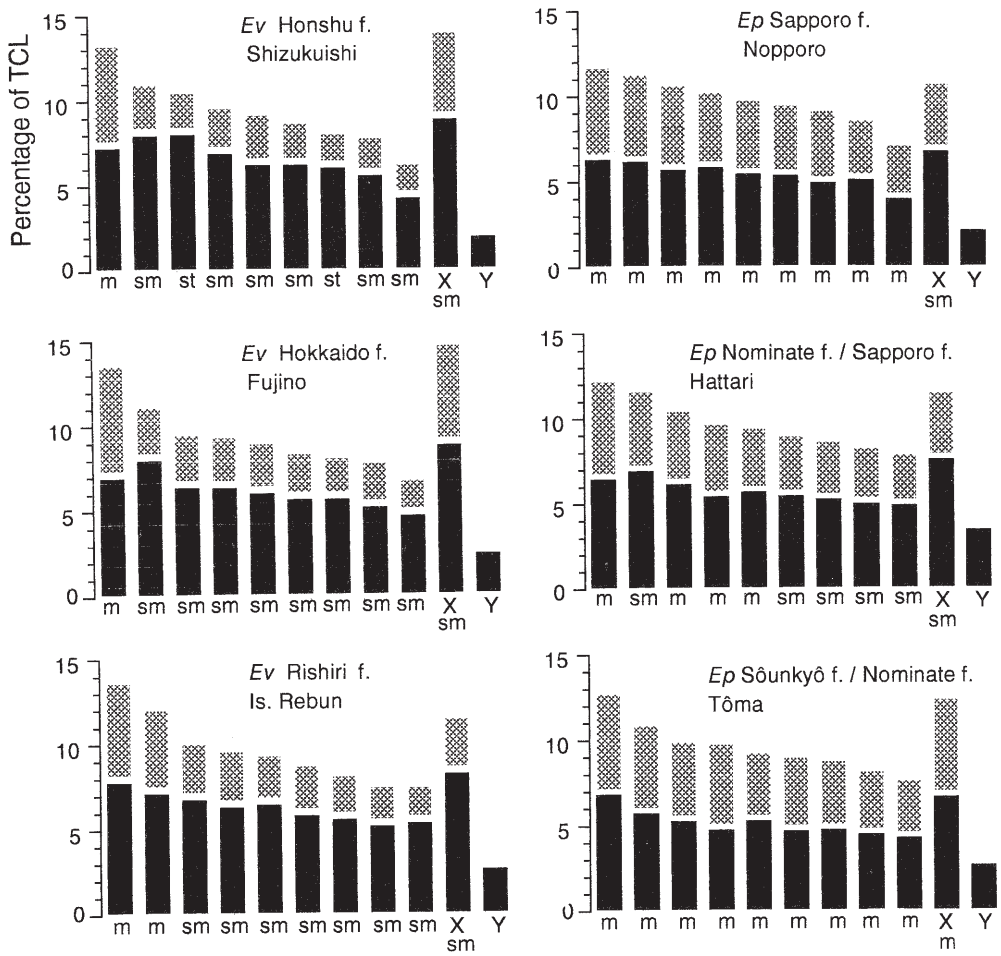

FIG. 4. Idiograms of *Epilachna vigintioctomaculata* (*Ev*) and *E. pustulosa* (*Ep*), based each on measurements for five chromosomal spreads per each population. Kamuikotan population of *E. pustulosa* showed same karyotype as that from Hattari. m=metacentric, sm=submetacentric, st=subtelocentric. Note that the representation of heterochromatic arms (crosshatched) in this figure is extremely simplified. It does not mean whole segments of those arms consist solely of heterochromatin.

the so-called Western Tokyo form, which is found in potato fields of southwestern Kantô district and southern Chûbu district, central Honshu, as a member of this species [16].

The autosomes almost invariably consisted of 9 pairs of metacentrics. The X is the first or the second largest meta- or submetacentric chromosome. Y/X ratios ranging from 0.17 to 0.32 do not significantly differ among populations (Kruskal-Wallis test, $P=0.13$). The general karyotypes are

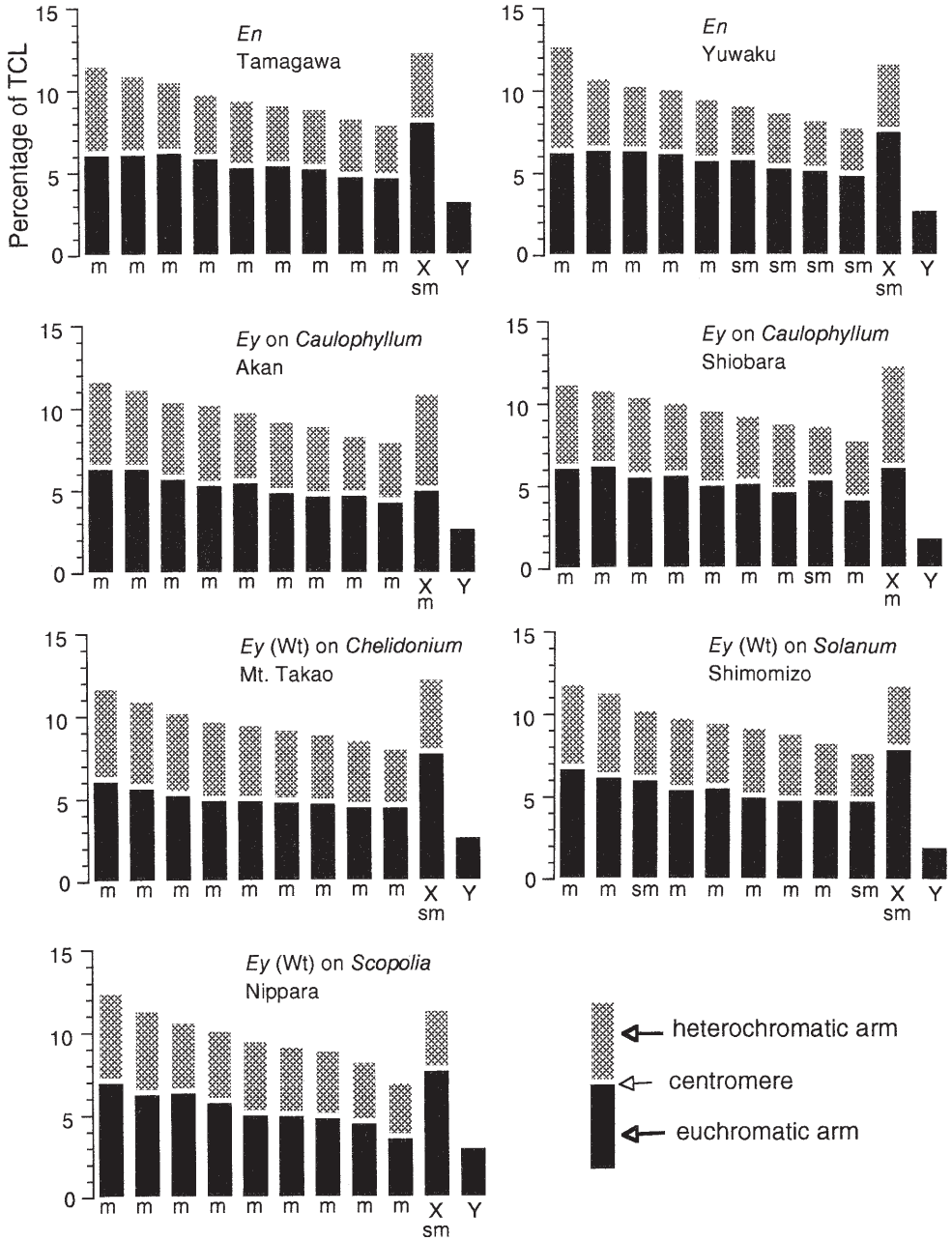

FIG. 5. Idiograms of two forms of *Epilachna niponica* (*En*) and five populations of *E. yasutomii* (*Ey*). Explanation as in Fig. 4.

TABLE 2. Similarity values (upper right) and similarity coefficients (lower left) of karyotypes for 14 populations of the *Epilachna vigintioctomaculata* complex

	Ev1	*Ev2*	*Ev3*	*Ep1*	*Ep2*	*Ep3*	*Ep4*	*Ey1*	*Ey2*	*Ey3*	*Ey4*	*Ey5*	*En1*	*En2*
Ev Honshu f. (Shizukuishi)		17	15	11	6	10	4	4	7	6	10	8	10	10
Ev Hokkaido f. (Fujino)	.85		16	10	8	9	5	4	5	6	8	7	13	10
Ev Rishiri f. (Is Rebun)	.75	.80		16	12	11	7	9	9	8	13	11	16	15
Ep Nominate/Sapporo f. (Hattari)	.55	.50	.80		20	18	15	14	14	16	14	20	19	19
Ep Nominate f. (Kamuikotan)	.30	.40	.60	1.00		17	19	17	17	16	19	18	19	19
Ep Sôunkyô/Nominate f. (Tôma)	.50	.45	.55	.90	.85		17	17	17	18	18	19	17	19
Ep Sapporo f. (Nopporo)	.20	.25	.35	.75	.95	.85		17	17	18	20	17	15	17
Ey (Akan)	.20	.20	.45	.70	.85	.85	.85		19	19	19	17	14	18
Ey (Shiobara)	.35	.25	.45	.70	.85	.85	.85	.95		18	19	17	14	19
Ey-Wt (Mt. Takao)	.30	.30	.40	.80	.80	.90	.90	.95	.90		19	18	13	19
Ey-Wt (Nippara)	.50	.40	.65	.70	.95	.90	1.00	.95	.95	.95		17	15	20
Ey-Wt (Shimomizo)	.40	.35	.55	1.00	.90	.95	.85	.85	.85	.90	.85		17	20
En (Kanazawa)	.50	.65	.80	.95	.95	.85	.75	.70	.70	.65	.75	.85		19
En (Tamagawa Spa)	.50	.50	.75	.95	.95	.95	.85	.90	.95	.95	1.00	1.00	.95	

Ev=*E. vigintioctomaculata*, *Ep*=*E. pustulosa*, *En*=*E. niponica*, *Ey*=*E. yasutomii*, Wt=Western Tokyo form.

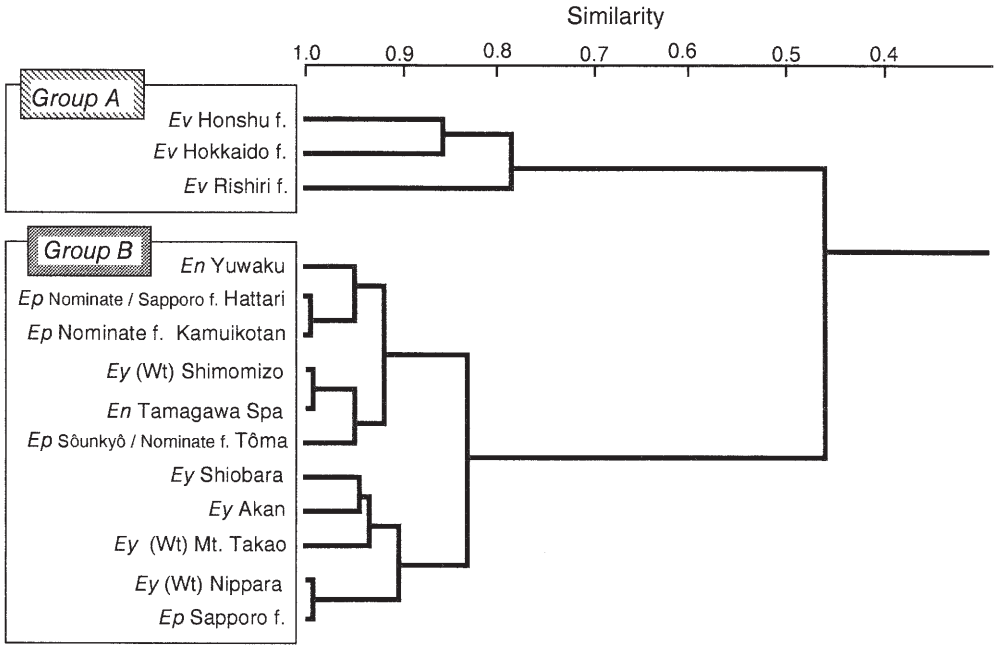

FIG. 6. Dendrogram resulting from UPGMA cluster analysis of the karyotypes of 14 populations. *Ev*=*E. vigintioctomaculata*, *En*=*E. niponica*, *Ep*=*E. pustulosa*, *Ey*=*E. yasutomii*, Wt=Western Tokyo form.

similar to each other (similarity coefficient ranges 0.85–0.95), although ratio of total length of heterochromatic arms to total length of euchromatic ones varied among the five populations (Kruskal-Wallis test, $P<0.05$).

Comparison of karyotypes among four species

Figure 6 represents a UPGMA dendrogram de-

rived from the similarity coefficients of karyotypes shown in Table 2. The procedure used in the present study to compare two karyotypes has an apparent defect. We may erroneously compare the nonhomologous pairs of chromosomes from two karyotypes, when some of the component chromosomes are very similar in size and shape. Nevertheless, resulted dendrogram was the one fairly compatible with the phylogeny of the complex inferred from the analyses of external morphology and/or host plant preference [12]. The karyotypes were classified into two major groups. One contained all the populations of *E. vigintioctomaculata,* and the other consisted of members of three other species, *E. pustulosa, niponica,* and *yasutomii.* Furthermore, the latter could be divided into two minor groups, one of which is comprised mostly of members of a single species *E. yasutomii.*

The principal component analysis based on the 21 measured karyomorphological characters (Table 3) offered similar results for the separation of the two major groups of *vigintioctomaculata* complex. Table 3 presents the loadings on the first two principal components. The first two components account for more than 70% of the variance. The first axis had high positive loadings for arm ratios of chromosomes Nos. 2 to 9 and relative lengths of chromosome No. 1 and sex chromosome X. The first component is also characterized by high negative loadings for lengths of chromosome Nos. 4–9, especially of Nos. 6–8. It is easy to understand that the arm ratios and the relative lengths of chromosomes contribute to the first principal component in opposite directions. By gaining longer heterochromatic segments, relative lengths of the relevant chromosomes would automatically increase, whereas the arm ratios (length of long arm/length of short arm) decrease. Hence, we interpret the first component as a factor that pertains to presence or absence of long heterochromatic segments in chromosomes Nos. 2–9, particularly in Nos. 3–9. The second principal component pertains positively to length of chromosome No. 2, and arm ratios in chromosomes X and No. 1 and negatively to length of the X chromosome. High scores on the second principal component represent populations with submetacentric and/or relatively small X or with chromosome No. 2 somewhat larger in relative length.

Figure 7 plots the positions of the populations on the first two principal components. It shows again a clear separation of the plots between *E. vigintioctomaculata* and the other three species for the axis of the first principal component. Among three populations of the former species, the Rishiri form was plotted on rather remote position from

TABLE 3. Loadings of the measured parameters on the first two principal components (PC)

	Parameter[1)]	PC1	PC2		Parameter[2)]	PC1	PC2
1	Ch No. 1: length	0.87	0.16	12	Ch No. 1: arm ratio	−0.03	0.66
2	Ch No. 2: length	0.30	0.83	13	Ch No. 2: arm ratio	0.87	−0.30
3	Ch No. 3: length	−0.44	0.41	14	Ch No. 3: arm ratio	0.90	0.07
4	Ch No. 4: length	−0.75	0.15	15	Ch No. 4: arm ratio	0.92	0.02
5	Ch No. 5: length	−0.78	0.35	16	Ch No. 5: arm ratio	0.93	0.25
6	Ch No. 6: length	−0.90	0.21	17	Ch No. 6: arm ratio	0.94	0.05
7	Ch No. 7: length	−0.96	0.05	18	Ch No. 7: arm ratio	0.97	0.09
8	Ch No. 8: length	−0.87	−0.26	19	Ch No. 8: arm ratio	0.90	0.10
9	Ch No. 9: length	−0.65	0.03	20	Ch No. 9: arm ratio	0.93	0.23
10	Ch X: length	0.75	−0.60	21	Ch X: arm ratio	0.28	0.71
11	Ch Y: length	−0.10	0.30				
					Explained variance (%)	59.6	13.1

[1)] The length is relative length to the TCL, total length of haploid chromosomes.
[2)] Arm ratio=length of long arm/length of short arm.

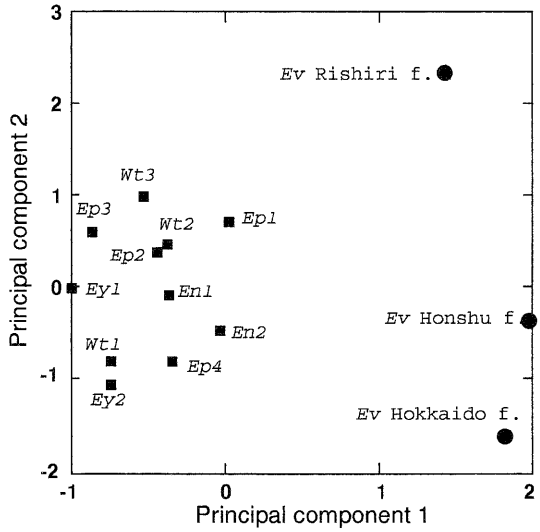

FIG. 7. Projection of 14 populations on the first two principal components. Circles=*E. vigintioctomaculata* (*Ev*), squares=other three species. *Ep*=*E. pustulosa*, *En*=*E. niponica*, *Ey*=*E. yasutomii*, *Wt*=Western Tokyo form of *E. yasutomii*. *Ep1*=A population with an intermediate phenotype between Nominate f. and Sapporo f. (Hattari), *Ep2*=Nominate f. (Kamuikotan), *Ep3*=Sapporo f., *Ep4*=Sôunkyô f., *En1*=Tamagawa Spa, *En2*=Yuwaku, *Ey1*=Akan, *Ey2*=Shiobara, *Wt1*=Mt. Takao, *Wt2*=Shimomizo, *Wt3*=Nippara.

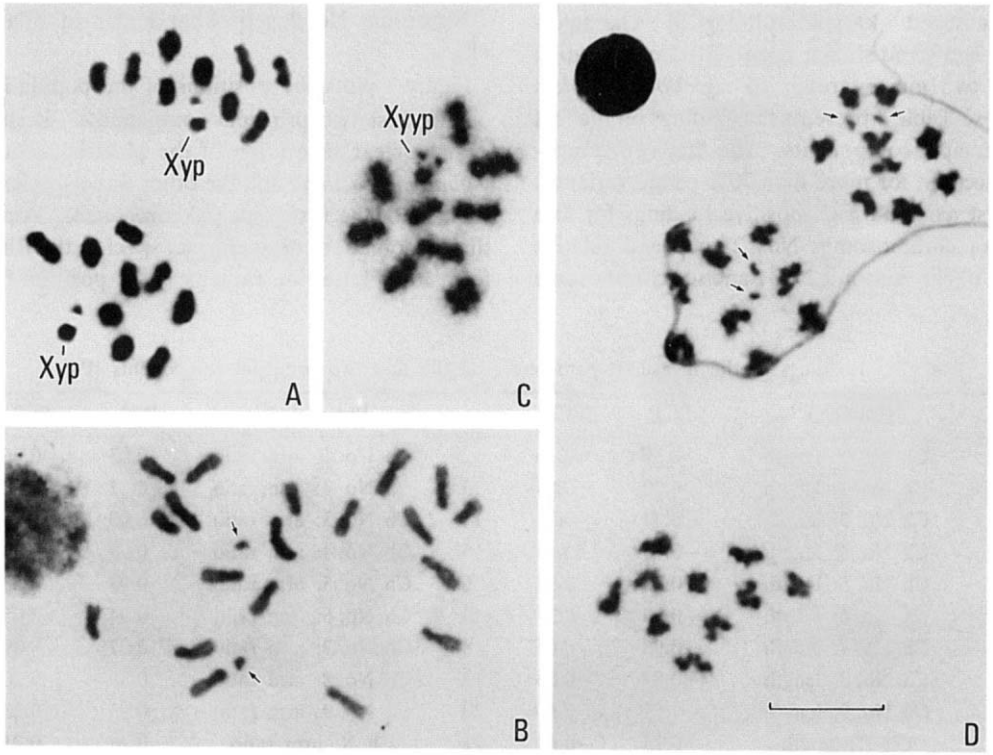

FIG. 8. Chromosomes of *E. pustulosa*. A, Two first meiotic metaphases showing normal Xy_p associations. B, Spermatogonial metaphase with two Y chromosomes (arrowed) (2n=21). C, First meiotic metaphase with an Xyy_p. D, Second meiotic metaphases showing two classes with two Ys (two cells above; Ys are arrowed), and without Y (bellow). Scale=0.01 mm. A-C: Hattari, D: Monbetsu.

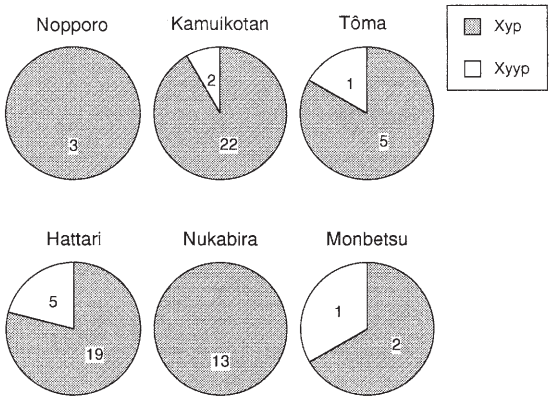

FIG. 9. Frequencies of males with Xyy_p and Xy_p in six populations of *E. pustulosa*. Numbers of individuals examined are also shown in each pie chart. Nopporo: Sapporo form. Hattari: a population with an intermediate phenotype between Nominate and Sapporo forms. Kamuikotan, Nukabira: Nominate form. Tôma: a population with an intermediate phenotype between Nominate and Sôunkyô forms. Monbetsu: Sôunkyô form.

the remaining two populations (the Honshu and the Hokkaido forms) along the second axis. On the other hand, 11 populations in the other three species were less variable along the second principal component axis. Two subgroups recognized in the populations of the group B in the cluster analysis (Fig. 6) also differ in the scores in the first principal component axis, but they cannot be separated clearly on this basis.

Supernumerary Y chromosomes

In males of *E. pustulosa,* supernumerary chromosomes were found with fairly high frequencies over wide geographical range (Table 1 and Figs. 8, 9). Those were small chromosomal fragments, approximately equal in size to the Y chromosome and were virtually indistinguishable from the Y. The number of the supernumerary chromosome retained per cell was always one if any. Neither males with more than one supernumerary chromosome nor females with a comparable one have been found. During meiosis in males, the supernumerary chromosome paired with X together with a Y, forming so-called Xyy_p association (Fig. 8C). At the second meiotic division, two classes of cells, one with two "Y"s and the other with no "Y" were observed (Fig. 8D). These facts indicate that the supernumerary is not a true B-chromosome which appears irrespective of sex, but a supernumerary Y chromosome derived probably from duplication of the Y.

Frequencies of males with a supernumerary Y in six populations of *E. pustulosa* ranged 0–33.3%, though there were no significant differences in the frequency between populations (>0.05, Fisher's exact probability tests for every pair of two populations). Among five males with Xyy_p found from Hattari population, one showed a mosaic of Xy_p and Xyy_p at meiosis and 2n equals 20 and 21 at mitotic division.

DISCUSSION

Karyotypic differentiation between E. vigintioctomaculata and other three species and its possible relevance to the reproductive isolation

Comparative studies of chromosomes of beetles belonging to the *vigintioctomaculata*-complex of *Epilachna* have been made by Yosida [44] and Takenouchi [36]. They found slight karyological differences between *E. vigintioctomaculata* and *E. pustulosa* at spermatogonial metaphase [44] and first meiotic metaphase [36]. However, their results *per se,* which were obtained from out-of-date methods like paraffin section or squash preparations, are not comparable with ours using current air-drying technique.

We successfully revealed an apparent differentiation in karyotype between *E. vigintioctomaculata* belonging to the group A and three other species of the group B (sensu Katakura [12]). It was shown that the major difference comes from addition of long heterochromatic segments in Nos. 3–9 chromosomes in the group B karyotypes (Fig. 8). Due to this alteration, those chromosomes in the group B show far more evident diphasic state than those in *E. vigintioctomaculata.* Diphasics are chromosomes with one arm heterochromatic and the other euchromatic. They have been found in several groups of beetles, such as *Chilocorus* of Coccinellidae [3–5, 29, 30], *Pissodes* (Curculionidae) [31, 32], and *Carabus* (Carabidae) [42]. Labelling with thymidine, Weber [42] and Ennis

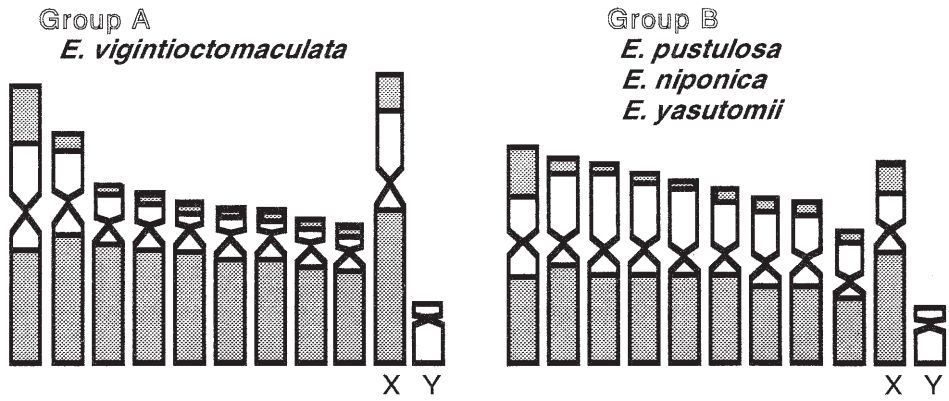

FIG. 10. Schematic representation of karyotypes of *E. vigintioctomaculata* and three other species of the group B. Distribution of heterochromatic (unshaded) and euchromatic (shaded) segments was inferred from C-banded karyotypes.

[5] demonstrated that heterochromatic arms in those diphasic chromosomes are late-replicating. Chromosome configurations which often look like letter Y, exhibited by most of chromosomes in the group B, also indicate the late replication of heterochromatic arms.

Autosomes Nos. 3–8 of *Epilachna vigintioctopunctata* (Fabricius), another *Epilachna* species widespread in southeast Asia, totally lack short arms consisting of heterochromatin (Tsurusaki et al. unpublished data). Furthermore, no typical diphasics have been found in several other species of *Epilachna* [1, 2], Tsurusaki et al. unpubl.). Therefore the exaggerated diphasic state is considered to be a synapomorphy shared by three species of the group B. The origin of these heterochromatic segments is unclear. However, gradual heterochromatinisation of euchromatic arms is unlikely. Comparison of the group B karyotypes with the karyotype of *E. vigintioctomaculata* suggests concurrent accretion of heterochromatic segments on short arms of autosomes Nos. 3 to 9 by tandem repetition of existent heterochromatin.

Although differentiation of karyotype between *E. vigintioctomaculata* and species of the group B in terms of the amount of heterochromatin seems to be profound, its relation to the speciation in the species-complex is uncertain. Takenouchi [36] failed to detect any cytological abnormality in spermatogenesis in male hybrids between *E. vigintioctomaculata* and *E. pustulosa*. Homologues from both genomes formed bivalents without any trouble. However, the quantitative difference of heterochromatin between the two groups might do have a substantial effect on their reproductive isolation.

It has been established that there are rather strong postmating reproductive isolation between *E. vigintioctomaculata* and three other species of the group B, although the premating (sexual) isolation is fairly incomplete [14, 18, 20]. On the other hand, postmating reproductive barrier among three species of the group B is far from complete [17, 19]. The postmating isolation between *E. vigintioctomaculata* and members of group B is characterized by the fact that hatchability of those F_1 hybrids is extremely low, whereas viability of the F_1 hybrids after their hatching is considerably high. Katakura and his coworkers attributed the low hatchability in F_1 hybrid to the incompatibility between the female genital tract and heterospecific sperms in the *vigintioctomaculata* complex [14, 20]. However some other factors might also be responsible for the low hatchability.

It is well known that in early developmental stages of animals (e.g. before 11th cell division in *Drosophila*), m-RNAs needed for embryogenesis are maternally provided. On the other hand, genes carried by embryos do not initiate their transcription prior to a certain stage (usually gastrulation) of embryos [7, 9, 22, 27]. If it is true for

the present species of *Epilachna,* the rate of cell divisions in early embryogenesis, which is expected to be determined by maternal gene products, may not synchronize with the rate of chromosome replication in hybrid zygote which is comprised of both early-replicating monophasic chromosomes and diphasics with late-replicating heterochromatic arms. Thus, lethality in F_1 hybrid embryos resulting from heterospecific crossings might be attributable to such a discordance between the rate of cell division and that of chromosome replication. On the other hand, after the stages when zygotic mRNAs sufficiently replaced maternal ones, synchronization of cell division and chromosome replication would be restored and it would not disturb embryogenesis at all in later stages and during postembryonic development (Fig. 11).

This hypothesis has an advantage for parsimonious explanation of some outstanding features derived from the heterospecific crossings of the *vigintioctomaculata*-complex. First, it explains well the high viability during larval period in heterospecific hybrids when they survived embryogenesis. Second, it can also account for the amelioration of hybrid fitness in later filial generations [24]. Thirdly, it is also consistent with the presence of postmating reproductive isolation between *E. vigintioctomaculata* and the group B and absence of it among the species of the latter, since only *E. vigintioctomaculata* deviates from the other three species of the complex in karyological features concerning heterochromatic amounts.

Claims that constitutive heterochromatin may have played an important role in evolution and speciation in organisms have been appeared several times (e.g. [6, 26, etc.]; see John [8] for the

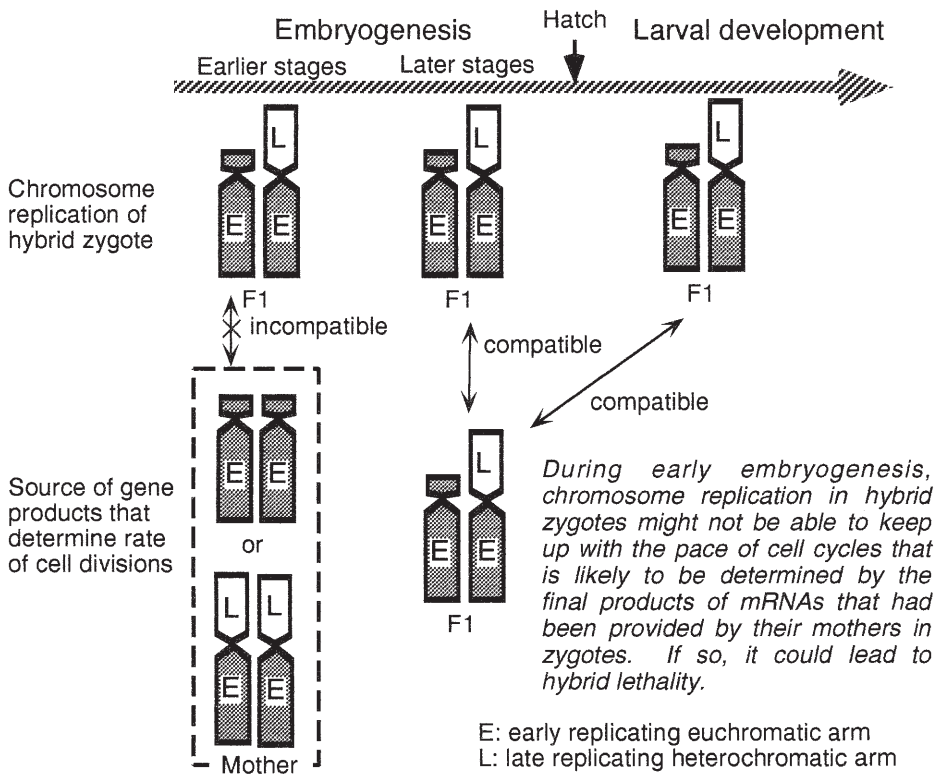

FIG. 11. A hypothetical model that explains hybrid lethality during embryogenesis of F_1 hybrid between *E. vigintioctomaculata* and other three species belonging to the group B. Chromosomes derived from both *E. vigintioctomaculata* and other three species are respectively depicted as monophasics (E) and diphasics (L and E) for the simplicity.

critical review). Those claims rest on the two possible effects of heterochromatin: (1) the presence of heterochromatin might facilitate structural rearrangements and might eventually lead to reproductive isolation; (2) species specificity of heterochromatin will confer an effective mechanism for postmating isolation if it prevents pairing of homologous chromosomes at meiosis [8]. Our idea is different from those formerly presented, for it postulates incompatibilities between the rate of chromosome replication that is determined by zygotic chromosomal structure *per se* and the rate of cell divisions determined by maternal gene products. We believe that our hypothesis deserves to be tested in the future study in closely related species with similar karyotype differentiation, as well as in the *vigintioctomaculata*-complex.

Karyotype differentiation within species

E. vigintioctomaculata. Both the cluster analysis and the principal component analysis for the karyotypes revealed apparent interpopulational variation in this species. Of the three populations, Is. Rebun population of the Rishiri form deviated to a large extent from the other two populations, each of which represents the Hokkaido form and the Honshu form, respectively. These results do not accord with those obtained from the analyses in external morphology, where no significant differences were found in external characters between the Rishiri form and the Honshu form, although the Hokkaido form conspicuously deviates from these two in the elytral shape [12]. The occurrence of apparent interspecific differentiation in karyotypes might suggest the longer evolutionary history of *E. vigintioctomaculata* in Japanese Island than ever been thought. It is premature, however, to conclude that the differences found in the karyotypes correspond well with the delimitation of three geographic races of the species, since each of the three forms was represented by only one population in the present study. Further study is needed to delineate the situation.

Three species of the group B. We failed to detect stable differences in the karyotypes among the species. However there were some noteworthy trends: In the cluster analysis, populations belonging to the group B were divided into two clusters, one consisting largely of populations of *E. yasutomii* (Ey) including the so-called Western Tokyo form and the other including two species, *E. niponica* (En) and *E. pustulosa* (Ep) (Fig. 6). The result of the principal component analysis suggests that this dichotomy is mainly based on the slight difference in arm ratios of autosomes Nos. 2 to 9 between most populations of *E. yasutomii* and those of the other two species. There is a tendency that short arms of those autosomes are fairly long in *E. yasutomii,* giving those chromosomes metacentric appearances.

Supernumerary Y in E. pustulosa

In the first meiotic division, X and Y sex chromosomes in most males of the *vigintioctomaculata* complex form a typical Xy_p bivalent, probably by the end-to-end association as revealed in a south American congener, *E. paenulata* [2]. However, an Xyy_p association was also found in some males from various populations of *E. pustulosa.* It is obvious that the Xyy_p association was derived from an addition of a supernumerary Y in those males. Comparison of the size and shape of the two Y chromosomes suggests that the supernumerary Y emanates from the duplication of Y. Y chromosomes, especially supernumerary Y's, have been so often treated as candidates for ancestors of B chromosomes [10]. However, in the present cases, the two Y chromosomes are always incorporated in the Xyy_p association, and no such chromosomes freed from the association just like true B chromosomes, were observed.

Effects of the supernumerary Y on the external morphology and/or life history characteristics are unclear. It is unlikely that its presence alters those phenotypic characters, since Y *per se* appears to be heterochromatic and genetically inactive. However, we have not yet made direct comparisons based on detailed measurements. It might also have effects on sex ratios as exemplified in dermestid beetles [28, 33]. Again, however, at present we have no data to test this possibility in *E. pustulosa.*

A structure similar to the Xyy_p has also been reported by Takenouchi [37] in four out of five males of *E. niponica* (identified as *E. pustulosa* in his paper) collected from Naeba, Niigata prefec-

ture, central Honshu. However, he interpreted the case as Xy_p in which the metacentric Y chromosome has a centric gap showing negative heteropycnosis , since no cells with two Y chromosomes were found at 2nd meiotic division. Consequently, it is difficult to conceive that this unusual case in *E. niponica* represents a precursory stage of true Xyy_p in *E. pustulosa*. Occurrence of the Xyy_p may be an autapomorphy of *E. pustulosa* alone, although Takenouchi's observation urges further study concerning the sex chromosome compositions in the whole members of the species-complex.

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APPENDIX

Measurements of chromosomes for 14 populations of the *Epilachna vigintioctomaculata* complex

RL=relative length of each chromosome to TCL, standing for total chromosome length for haploid genome. Means with the standard errors are presented for 11 chromosomes with following order: autosomes Nos. 1–9, X and Y sex chromosomes. AR=average arm ratios (lengths of long arm/short arm) with SEs for autosomes Nos. 1–9 and X chromosome.

1. *Epilachna vigintioctomaculata* (Honshu form), Shizukuishi (n=5). RL: 13.4±0.35 / 11.2±0.25 / 10.7±0.19 / 9.8±0.17 / 9.3±0.12 / 8.9±0.16 / 8.2±0.09 / 8.0±0.08 / 6.4±0.22 / 14.1±0.43 / 2.2±0.16. AR: 1.3±0.05 / 3.0±0.44 / 3.8±0.76 / 2.9±0.35 / 2.3±0.17 / 2.7±0.17 / 3.3±0.33 / 2.8±0.25 / 2.5±0.39 / 1.8±0.09.

2. *Epilachna vigintioctomaculata* (Hokkaido form), Fujino, Sapporo (n=5). RL: 13.7±0.48 / 11.3±0.43 / 9.7±0.20 / 9.5±0.18 / 9.2±0.38 / 8.5±0.14 / 8.3±0.14 / 8.0±0.16 / 7.0±0.34 / 14.8±0.39 / 2.7±0.35. AR: 1.1±0.03 / 2.9±0.42 / 2.4±0.48 / 2.4±0.43 / 2.2±0.11 / 2.3±0.23 / 2.5±0.08 / 2.3±0.38 / 2.6±0.33 / 1.6±0.22.

3. *Epilachna vigintioctomaculata* (Rishiri form), Motoji, Is. Rebun (n=5). RL: 13.8±0.65 / 12.2±0.32 / 10.3±0.22 / 9.8±0.16 / 9.6±0.12 / 8.9±0.22 / 8.3±0.09 / 7.7±0.32 / 7.7±0.09 / 11.7±0.64 / 2.9±0.17. AR: 1.4±0.09 / 1.6±0.16 / 2.4±0.37 / 2.1±0.21 / 2.5±0.38 / 2.2±0.24 / 2.6±0.56 / 2.5±0.25 / 2.9±0.38 / 2.8±0.22.

4. *Epilachna pustulosa* (Sapporo form), Nopporo (n=5). RL: 11.8±0.20 / 11.4±0.22 / 10.8±0.18 / 10.4±0.20 / 9.9±0.06 / 9.6±0.10 / 9.3±0.22 / 8.7±0.13 / 7.3±0.15 / 10.9±0.13 / 2.4±0.16. AR: 1.2±0.08 / 1.3±0.13 / 1.2±0.09 / 1.4±0.13 / 1.3±0.10 / 1.4±0.19 / 1.3±0.12 / 1.6±0.17 / 1.5±0.26 / 1.9±0.26.

5. *Epilachna pustulosa* (A population with an intermediate phenotype between Nominate form and Sapporo form), Hattari (n=5). RL: 12.3±0.29 / 11.7±0.17 / 10.6±0.20 / 9.8±0.16 / 9.6±0.10 / 9.1±0.20 / 8.8±0.15 / 8.4±0.21 / 8.0±0.17 / 11.6±0.52 / 3.6±0.07. AR: 1.2±0.07 / 1.7±0.35 / 1.5±0.07 / 1.4±0.11 / 1.6±0.18 / 1.8±0.32 / 1.7±0.15 / 1.7±0.26 / 1.8±0.11 / 2.2±0.41.

6. *Epilachna pustulosa* (Nominate form), Kamuikotan (n=5). RL: 12.5±0.15 / 11.2±0.30 / 10.5±0.32 / 10.1±0.19 / 9.6±0.13 / 9.5±0.15 / 9.1±0.12 / 8.7±0.09 / 7.6±0.35 / 11.3±0.67 / 3.2±0.37. AR: 1.2±0.07 / 1.4±0.11 / 1.5±0.44 / 1.6±0.36 / 1.4±0.04 / 1.4±0.14 / 1.5±0.12 / 1.7±0.06 / 1.6±0.25 / 2.3±0.27.

7. *Epilachna pustulosa* (A population with an intermediate phenotype between Nominate form and Sôunkyô form), Tôma (n=5). RL: 12.9±0.56 / 11.0±0.31 / 10.1±0.18 / 9.9±0.16 / 9.4±0.12 / 9.1±0.13 / 8.9±0.17 / 8.3±0.16 / 7.8±0.22 / 12.5±0.80 / 2.9±0.31. AR: 1.3±0.19 / 1.2±0.08 / 1.2±0.15 / 1.0±0.09 / 1.5±0.25 / 1.2±0.08 / 1.3±0.04 / 1.3±0.12 / 1.4±0.08 / 1.3±0.16.

8. *Epilachna niponica,* Tamagawa Spa, Akita Pref. (n=4). RL: 11.6±0.39 / 11.0±0.22 / 10.7±0.34 / 9.9±0.17 / 9.5±0.09 / 9.3±0.14 / 9.0±0.11 / 8.4±0.12 / 8.1±0.13 / 12.4±0.72 / 3.4±0.35. AR: 1.2±0.17 / 1.3±0.16 / 1.6±0.24 / 1.6±0.28 / 1.4±0.11 / 1.6±0.16 / 1.5±0.19 / 1.5±0.33 / 1.6±0.19 / 2.0±0.27.

9. *Epilachna niponica,* Yuwaku, Kanazawa (n=5). RL: 12.8±0.31 / 10.9±0.23 / 10.4±0.29 / 10.2±0.24 / 9.6±0.14 / 9.2±0.18 / 8.8±0.18 / 8.3±0.12 / 7.9±0.16 / 11.8±0.64 / 2.9±0.19. AR: 1.0±0.10 / 1.6±0.28 / 1.7±0.12 / 1.6±0.12 / 1.6±0.20 / 1.9±0.18 / 1.7±0.18 / 1.9±0.29 / 1.9±0.26 / 2.0±0.23.

10. *Epilachna yasutomii,* Akan, Hokkaido (n=5). RL: 11.8±0.26 / 11.3±0.09 / 10.5±0.06 / 10.4±0.08 / 9.9±0.15 / 9.4±0.21 / 9.1±0.20 / 8.5±0.17 / 8.1±0.24 / 11.0±0.80 / 2.8±0.19. AR: 1.2±0.07 / 1.4±0.10 / 1.3±0.14 / 1.1±0.06 / 1.3±0.02 / 1.2±0.04 / 1.2±0.09 / 1.4±0.10 / 1.2±0.07 / 0.9±0.07.

11. *Epilachna yasutomii,* Shiobara (n=5). RL: 11.3±0.25 / 11.0±0.13 / 10.5±0.13 / 10.2±0.25 / 9.7±0.16 / 9.4±0.15 / 8.9±0.12 / 8.8±0.13 / 7.9±0.20 / 12.4±0.74 / 2.0±0.25. AR: 1.2±0.11 / 1.5±0.35 / 1.2±0.07 / 1.4±0.18 / 1.2±0.10 / 1.3±0.16 / 1.2±0.09 / 1.9±0.34 / 1.2±0.22 / 1.0±0.09.

12. *Epilachna yasutomii* (Western Tokyo form), Shimomizo, Hachiôji, Tokyo (n=5). RL: 12.0±0.26 / 11.4±0.13 / 10.4±0.08 / 9.9±0.10 / 9.6±0.07 / 9.4±0.14 / 9.0±0.19 / 8.5±0.11 / 7.9±0.21 / 12.0±0.23 / 2.2±0.10. AR: 1.4±0.09 / 1.3±0.04 / 1.7±0.42 / 1.3±0.08 / 1.5±0.06 / 1.3±0.18 / 1.3±0.09 / 1.5±0.22 / 1.7±0.13 / 2.2±0.30.

13. *Epilachna yasutomii* (Western Tokyo form), Mt. Takao, Tokyo (n=5). RL: 11.8±0.42 / 11.1±0.32 / 10.3±0.21 / 9.8±0.21 / 9.6±0.16 / 9.3±0.06 / 9.0±0.08 / 8.6±0.05 / 8.1±0.11 / 12.3±0.61 / 2.8±0.06. AR: 1.1±0.08 / 1.1±0.10 / 1.1±0.11 / 1.1±0.07 / 1.1±0.10 / 1.2±0.09 / 1.2±0.10 / 1.2±0.13 / 1.4±0.17 / 1.9±0.30.

14. *Epilachna yasutomii* (Western Tokyo form), Nippara, Tokyo (n=5). RL: 12.5±0.64 / 11.5±0.28 / 10.8±0.13 / 10.3±0.12 / 9.6±0.13 / 9.3±0.15 / 9.1±0.21 / 8.4±0.17 / 7.1±0.44 / 11.5±0.98 / 3.2±0.18. AR: 1.4±0.18 / 1.3±0.16 / 1.6±0.23 / 1.5±0.25 / 1.2±0.05 / 1.3±0.12 / 1.2±0.06 / 1.3±0.14 / 1.2±0.13 / 2.2±0.23.